

Microcalorimetric studies
of human erythrocytes
and lymphocytes

Julie Ama Ikomi-Kumm



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MICROCALORIMETRIC STUDIES OF HUMAN ERYTHROCYTES AND LYMPHOCYTES

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Abstract Methodological studies on the use of microcalorimetry for monitoring the metabolic activity in human blood erythrocytes and lymphocytes are described. Two types of microcalorimetric techniques were compared. Heat production rate (thermal power), an index of overall metabolism was determined in suspensions of erythrocytes and lymphocytes in plasma and in buffer under various experimental conditions. The determined rates of heat production in the erythrocyte suspensions were evaluated with respect to contributions from the activities of two enzyme systems involved in energy production and utilisation. Determined values of heat production rates in erythrocytes from obese subjects and lymphocytes from patients with lymphoid disease indicate possible prognostic use of the technique of microcalorimetry in the clinical field.		
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This thesis is based on the following papers, which are referred to by the Roman numerals I-VII.

I. J. A. Ikomi-Kumm and A. E. Boyo

Heat Production by Human Erythrocytes in Vitro: a Study of Two Different Populations

IRCS Med. Sci. 5, 576, 1977

II. J. A. Ikomi-Kumm

The Effect of Cellular Flow and Viscosity on Heat Production by Human Erythrocytes Measured in Vitro by Flow Reaction Calorimetry
IRCS Med. Sci. 5, 320, 1977

III. J. A. Ikomi-Kumm

The Relationship between Heat Production Rate, Glycolysis and Na^+ , K^+ - ATPase Activity in Human Erythrocytes
In manuscript

IV. M. Monti and J. A. Ikomi-Kumm

Erythrocyte Heat Production in Human Obesity. Microcalorimetric Investigation of Sodium-Potassium Pump and Cell Metabolism
Metabolism, submitted

V. J. A. Ikomi-Kumm, M. Monti and I. Wadsö

Heat Production in Human Blood Lymphocytes. A Methodological Study.
Scand. J. clin. Lab. Invest., submitted

VI. L. Brandt, J. A. Ikomi-Kumm, M. Monti and I. Wadsö

Heat Production by Lymphocytes in Chronic Lymphocytic Leukaemia
Scand. J. Haematol. 22, 141, 1979

VII. M. Monti, L. Brandt, J. A. Ikomi-Kumm, H. Olsson and I. Wadsö

Metabolic Activity of Lymphoma Cells and Clinical Course in Non-Hodgkin Lymphoma (NHL)
Scand. J. Haematol. 27, 305, 1981.

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1. INTRODUCTION

Human blood cells extract energy from the degradation of simple substrates with concomitant production of heat. The heat produced in the course of cellular metabolism is not available for useful work under the isothermal conditions of the cell and represents a degree of inefficiency of energy transformation. In recent years, the rate of heat production has become a useful index of the complex reactions occurring in the multi-enzyme system of the cell (37). Since the advent of sensitive reaction calorimeters, "microcalorimeters", about two decades ago, microcalorimetry has increasingly found application in several areas of research on cellular systems. The technique has been applied to studies in various fields of microbiology, in clinical chemistry, and in the general clinical field (6, 27, 65).

The work of Levin and Boyo (31) in 1971, using the LKB flow microcalorimeter for monitoring heat production rates in human erythrocytes, was the earliest application of the new generation of microcalorimeters to the study of blood cell metabolism. Soon after, Levin (32) extended his studies to leukocyte and platelet activity in health and during thyroid dysfunction. Then Boyo et al. (8) showed that the flow technique could be used to monitor the abnormal metabolism of whole blood and erythrocytes in some hemoglobinopathies. Concurrently, Levin (33) and Monti & Wadsö (45), using flow and static microcalorimeters respectively, showed that erythrocytes from different types of anemic patients produced heat at increased rates in comparison to erythrocytes from healthy subjects. A later study by Levin et al. (35) related heat production rates in human erythrocytes

to their metabolism of glucose and amino acids. The study shed some light on the sources of the heat produced at the molecular level. Using a flow microcalorimeter, Minakami & de Verdier (43) determined heat production rates in erythrocytes under various metabolic conditions with simultaneous determination of lactate and other metabolites and discussed their data in relation to the thermodynamics of erythrocyte glycolysis.

With the introduction of the static ampoule calorimeter (69) of higher sensitivity and improved precision of sampling, Bandmann et al. (5) determined heat production rates in whole blood, plasma and its cellular components, erythrocytes, platelets and leucocytes (lymphocytes, monocytes and polymorphonuclear leucocytes, PMN). They showed that the heat production rate in whole blood was in agreement with the value obtained from the sum of the rates in plasma and the cellular components of blood. Of these cellular components, only erythrocytes and thrombocytes showed acceptable coefficients of variation in heat production rates, about 7% and 14% respectively. Lymphocytes and PMN showed the highest, 64% and 29% respectively. It became clear at this stage that further methodological studies directed at gaining more information on inherent systematic errors and their elimination were necessary. Some of the necessary methodological studies on erythrocytes and platelets have since been carried out and reported (46, 47, 49). In these studies, the influence of pH temperature and glucose concentration on heat production rates in erythrocyte and platelet suspensions were examined using the flow and static versions of the calorimeter.

The experience gained from these methodological studies by the static ampoule technique has been applied in further calorimetric studies of erythrocytes in hyperthyroidism (48), in uremia (50), and in liver disease (51). Similar application to platelet study indicated decreased platelet metabolism in patients with prosthetic heart valves (53). Basic methodological studies on the PMN, however, because of their high reactivity to a wide range of substances, are lacking. The early attempts by Levin, using the flow microcalorimeter, to investigate some functional aspects of leucocyte mixtures in health and in disease (34, 36) were hampered mainly by problems of adhesion of the phagocytic cells to the flow lines of the microcalorimeter. Recently, Eftimiadi and Rialdi (13), using an LKB flow microcalorimeter with some modifications and at high flow rate, reported simultaneous determinations of heat production rates (power values) and some crucial metabolites in suspensions of neutrophils in the presence of various chemical activators. Their data demonstrated the value of combining biochemical analysis with calorimetrically determined power values for gaining information on the heat contributions from reactions recognised for the metabolic bursts in activated neutrophils. Loike et al. (41) also recently reported simultaneous biochemical and calorimetric studies on murine macrophages using the differential scanning microcalorimeter over a temperature range of 10 - 37 °C. From determined heat quantities and rates of lactate production in the presence and absence of some inhibitors of metabolism, they estimated the enthalpy contributions from glycolysis. The present trend in calorimetric studies of cellular systems is clearly towards obtaining accurate information at the molecular level on specific aspects of cellular metabolism.

Methodological studies, aimed at defining optimum in vitro conditions for achieving this objective are without doubt imperative.

2. AIMS OF THIS STUDY

This study is part of a long-range project aimed at developing the microcalorimetric technique for the study of cellular systems. Some of the methodological problems indicated by earlier calorimetric determinations of heat production rates in blood cells are re-examined, with emphasis on erythrocytes and lymphocytes. Development of experimental procedures aimed at reducing systematic errors arising from methods of cell preparations and procedures of calorimetric measurements were deemed necessary for further application of microcalorimetry to thermodynamic studies at the molecular level and to studies in the clinical field. The following are some of the critical problems left unresolved by earlier work and investigated in this study:

- i Large intersubject and intersample variation in heat production rates, especially for lymphocytes and PMN.
- ii Variability of heat production profiles (power-time curves) and their dependence on sample size.
- iii Variability in heat production rate (power) per cell with cell density, the so-called "crowding effect".
- iv Lack of correspondence between the rate of glucose consumption and power values in erythrocyte suspensions.
- v Evident adhesion of cells to flow tubings and walls of reaction vessels of the calorimeter.

- vi Dependence of heat production rates in erythrocyte suspensions on the flow rate of the suspension in flow microcalorimetry.

The apparent constancy of heat production rates in erythrocytes of healthy subjects under defined experimental conditions and the obvious possibility of using calorimetrically determined values as indices of metabolism in health and disease have given us a sustained impetus for further studies.

3. NOMENCLATURE

In recent times, the S.I. units (Le Système International d'unités), symbols and terminology have been recommended for use in biothermodynamics by the Interunion Commission in Biothermodynamics (42). These recommendations have been followed in the recent part of this work.

The unit for heat energy is the joule (J). The rate of heat production or thermal power (symbol P) is expressed in the unit joule per second (J/s) or watt (W). In calorimetric studies of cellular systems, the power is usually expressed in relation to commonly used data in haematology. Thus, for erythrocytes the power is expressed in watt per liter of packed cells (W/l). For other cell types, polymorphonuclear leucocytes, lymphocytes and platelets, the power is expressed in watt per cell (W/cell).

The molar enthalpy change, ΔH , for substrate metabolised, has the

unit joule per mol (J/mol). ΔH is a negative quantity for exothermic reactions, and the determined rates of heat production are therefore negative quantities. The calorimetric voltage difference generated by the heat produced in blood cell suspensions and monitored over a time interval is recorded as a voltage-time curve. Such curves have been converted into power-time curves by suitable calibrations.

In the earlier calorimetric studies, the calorimetric curves were denoted as thermograms, equivalent to the term "power-time curves" of recent studies. Also, the term heat production rate had both a qualitative and a quantitative meaning, with the units, calorie per hour (cal/h) or joules per hour (J/h) in the latter case, $1 \text{ cal/h} = 1.162 \text{ mJ/s} = 1.162 \text{ mW}$.

4. SOME NOTES ON BLOOD CELLS

For our calorimetric experiments, 40 ml of venous blood, readily available by venepuncture, offer an abundance of cells, over 160×10^9 cells, comprising erythrocytes, polymorphonuclear leucocytes (PMN), monocytes, lymphocytes and platelets.

4.1 Erythrocytes

The structure, chemical composition and metabolism of each cell type are adapted to its function. Thus, for the erythrocyte the large amounts of hemoglobin are adapted for oxygen and carbon dioxide transport. The shape and viscoelastic properties are adaptations for easy passage through fine capillaries. Its low oxidative metabolism

and the anaerobic nature of its principal metabolic pathway for generating energy from simple substrates are adaptations for economy of oxygen transport.

In health, the erythrocytes of peripheral blood are mature, biconcave cells having no nucleus, no mitochondria and no ribosomes. A semi-permeable membrane separates intracellular hemoglobin and a complex of enzyme systems and ions from the extracellular fluid, plasma. ATP is generated from the degradation of simple sugars, predominantly glucose, via the Embden-Meyerhoff anaerobic pathway. In the sequence of reactions characterising this pathway, termed glycolysis, one mole of glucose is anaerobically degraded to 2 moles of lactic acid, with a net production of 2 moles of ATP. This aspect of erythrocyte metabolism has been investigated by microcalorimetry in a number of studies, e.g. (35, 43). The ATP produced in glycolytic reactions is coupled to the transport of Na^+ and K^+ against their electrochemical gradients. About 10% of the glucose metabolised by the erythrocytes goes through an oxidative pathway, the pentose phosphate pathway (PPP), and results in the production of CO_2 and reduced nicotinamide adenine dinucleotide phosphate, NADPH, a molecule of high reductive potential necessary for keeping sulphydryl groups of enzymes and membrane components in the reduced state and for combatting oxidant stress. In the presence of electron acceptors such as methylene blue or other oxidant compounds, this pathway can be greatly stimulated (9). By microcalorimetry, stimulation in the presence of methylene blue has been shown to lead to large increases in heat production rates (43, 52).

Studies of erythrocyte metabolism are abundant, a situation favoured by the simplicity of its metabolism. In recent years, both qualitative and quantitative models of glycolysis in erythrocytes have been constructed and tested, see e.g. (4, 24, 59) in attempts to understand the kinetics and mode of regulation of the multi-enzyme system of the erythrocyte. The experimental verifications of the quantitative model of Ataullakhanov et al. (3, 4) confirm earlier findings on the enzyme kinetics and regulation of glycolysis in the erythrocyte (24, 56, 70). The model proposes the dependence of the rate of glucose consumption on ATP concentrations, termed the characteristics of glycolysis. Experiments based on this model show that the dependence is bell-shaped, with a negative correlation between ATP concentration and rate of glucose consumption in the physiological range (3). This type of dependence allows for the stabilisation of the level of ATP in the cell, the mechanism of which is likely to contribute significantly to the power observed for erythrocytes.

4.2 Lymphocytes

Lymphocytes are usually classified morphologically as small, intermediate and large, with diameter (7-12) μm . In blood films stained with the commonly used stains (Giemsa and Grünwald-Wright), the lymphocyte appears as a round or oval cell with a small rim of pale blue and diffusely granular cytoplasm. Its nucleus of dense chromatin and RNA-positive material appears purple-blue. Electron microscopy reveals mitochondria, non-aggregated ribosomes, poorly developed Golgi apparatus and sparse and smooth endoplasmic reticulum in the cytoplasm.

Human lymphocytes comprise a heterogeneous population of cells having a coordinated immune function in the defence of man against foreign substances and infective agents. In health, this immune function is mainly that of surveillance and recognition of undesirable agents. In cooperation with the phagocytes (monocyte and macrophage), these agents are eliminated in reactions that constitute the immune response.

The small lymphocyte is a non-dividing cell and represents about 90% of peripheral blood lymphocytes. In the presence of a variety of substances, notably lecithins, subgroups of lymphocytes are stimulated and undergo transformation resulting in cell division and formation of blast cells (blastogenesis). The changes leading to blastogenesis may be observed morphologically as increases in cell size, in basophilia of cell cytoplasm, and compactness of nuclear chromatin. At the biochemical level the events accompanying blastogenesis include: increased glycolysis; increased RNA, DNA, protein synthesis; increased phospholipid fatty acid turnover; increased fluxes of Na^+ , K^+ , Ca^{2+} across the cell membrane; and increased thymidine uptake. The differential responses of the subgroups to different agents have been extensively utilised for assessing the immune status of lymphocytes in a number of in vitro systems (40). The works of Krakauer et al. (29) and Górski et al. (17) were attempts to extend the technique of microcalorimetry to this area of lymphocyte function.

Lymphocytes have been conveniently divided into two subpopulations, B (bone-marrow derived) and T (thymus derived). Detailed information on the different subgroups, their classification,

their methods of isolation, identification and enumeration are discussed in the report of a workshop sponsored by the World Health Organisation and the International Agency for Research on Cancer (71). B lymphocytes secrete and bear the immunoglobulin, SmIg, on their membrane surface. Using immunofluorescence techniques, this surface marker may be used to isolate and quantify the relative number of the subset in a lymphocyte population. T cells do not carry SmIg molecules but form rosettes with sheep erythrocytes (E-rosettes). This reaction constitutes the T-cell marker. Other subgroups carrying no markers or both markers of B and T cells are also described in the report (71).

In lymphoproliferative disease, lymphocyte surface markers and their patterns of expression are being increasingly used both for classification and prognostic purposes. The pattern emerging from various studies is increase in the relative number of tumor cells with B cell characteristics in various types of malignancies and reduction in the pool of T cells which normally regulate lymphoid cell differentiation. This has been found to be the case in both non-Hodgkin lymphoma (NHL) and in chronic lymphatic leukemia (CLL), (30, 39, 60) although in the latter case dominant T cell expression was also found (60). In B cell lymphoproliferative disorder, increased levels of the cell membrane protein β_2 -microglobulin are generally found, e.g. see (18). In malignant lymphoma and CLL, serum- β_2 -microglobulin levels seem to correlate with tumor cell mass (2, 63), and with poor prognosis in NHL (18). The possible use of microcalorimetry as an aid in the prognosis of NHL and understanding

of the pathophysiology of CLL are discussed in Papers VII and VI, respectively.

5. CALORIMETRY AND INSTRUMENTATION

Modern microcalorimeters are sensitive instruments which can be used for the measurement of the heat produced or absorbed in the reactions taking place in simple defined systems as well as in the complex multicomponent systems of e.g. living cells. The scope of application of calorimetric techniques in biological systems and the range of available instruments are described in recent reviews (6, 27, 65).

There are two basic types of calorimeters, the adiabatic and the isothermal. In the former there is no exchange of the heat produced in the reaction vessel with the surrounding one, and the resulting temperature rise in the reaction vessel is monitored. In the isothermal calorimeter, the heat of reaction is quantitatively transferred at constant temperature from the reaction vessel to the surrounding heat sink. Most of the calorimeters used for cellular studies are of the isothermal type based on the heat conduction principle. The heat of reaction is conducted to the heat sink through a series of thermopiles which serve as thermal meters with voltage outputs. As the thermopiles have high heat conductivity, the instruments work under nearly isothermal conditions.

In calorimetric work, the choice of instrumentation depends on the nature and kinetics of the reaction to be investigated and on the

information required. Since the biochemical reactions occurring in blood cell suspensions are slow, the heat conduction type of microcalorimeter has been the choice in most of the measurements of heat production rates in blood cells. For such slow reactions, the thermopile voltage, V , can be assumed proportional to the rate of heat flow, $\frac{dq}{dt}$

$$V = \text{constant} \frac{dq}{dt}$$

Under steady state conditions, the rate of heat flow is equal to the power, P , evolved by the sample.

$$P = \text{constant } V$$

For non-steady state conditions, e.g. such as obtain during fast chemical reactions, the voltage-time curves will lag behind the input power-time curves due to the thermal inertia of the systems. Using the frequency response method of Randzio et al (58), a dynamic correction can be applied to obtain the true power-time curve.

In the studies presented in this report, three versions of the heat conduction microcalorimeters were used. In the first phase of the studies on erythrocytes (Papers I, II), an LKB flow microcalorimeter, 10700-1 (LKB, Bromma, Sweden), of the type described by Monk et al (44), was used. This was the type of calorimeter used by Minakami et al (43), Levin et al (32, 33, 34, 35, 36) and by Eftimiadi et al (13), in their blood cell experiments. For later

studies (Papers III-VII), two nearly identical static ampoule calorimeters, described by Wadsö (69) and used by Monti et al in their earlier blood cell studies, e.g. (46, 49, 50), were used. In the last phase of the studies (Paper III), the above-mentioned static ampoule version and a three-channel prototype of the multichannel family of microcalorimeters, the LKB "Bio-Activity Monitor" (BAM), built at the Thermochemistry Laboratory, Chemical Center, Lund, were used. The construction and operation of this instrument have been described in detail (66). These different types of calorimeters, the flow type, the static ampoule type, and the three-channel static ampoule prototype, are designated as types A, B and C, respectively, in this report.

The flow reaction microcalorimeter - type A

Fig. 1 illustrates the essential features of the LKB flow microcalorimeter, type 10700-1. It consists of two calorimeter units and a centrally located heat exchanger coil unit enclosed in a metal heat sink. Embedded in each of the two calorimeter units are a mixing cell and a flow-through cell in good thermal contact with thermocouple plates. The metal heat sink is insulated and enclosed in an air-thermostatic bath maintained at 37°C . By means of a varioperpex pump between the sample and the flow-through measuring cell, the blood sample is introduced via the heat exchanger unit. During its flow through the heat exchanger unit, the sample assumes the temperature of the bath before entering the measuring cell. The flow lines from the pump into and out of the heat exchanger are made of teflon tubings. Both the heat exchanger coils and the flow-through cell tubing are made of 18 karat gold. The volume of the flow-through cell is 0.53 ml. The voltage output is registered with a Keithley nanometer voltmeter after amplification. The heat production rate, P , is registered as a deflection from a baseline established with water or physiological saline. This deflection is calibrated by application of electrical current through a precision resistor embedded at the base of the flow cell.

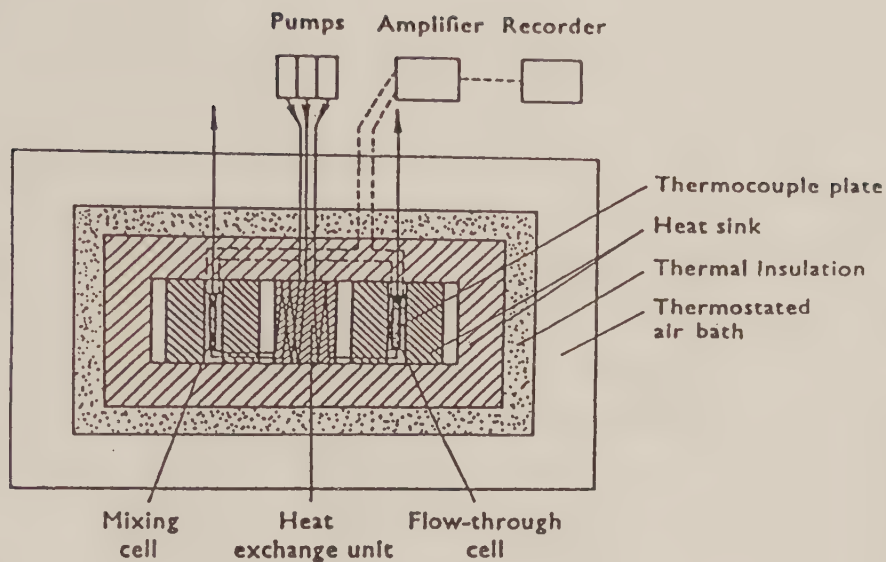


Fig. 1. The LKB flow reaction microcalorimeter.
Design according to Monk & Wadsö (ref. 44).
Reprinted from (ref. 68).



A flow-through cell of coiled gold;
component of the flow reaction microcalorimeter,
LKB Produkter, Bromma, Sweden.

The static ampoule microcalorimeter - type B

Fig. 2 illustrates the essential features of the static ampoule microcalorimeter. The main heat sink encloses two identical calorimeter units made of aluminium blocks. The heat sink is enclosed in a steel container and placed in a thermostated water bath at 37 °C. The reference and the reaction samples are enclosed in 1 ml stainless steel ampoules with rubber O-ring seals and introduced to the calorimeter reference and measuring cells respectively via steel tubes. The thermopiles are located at the base of each calorimeter unit and are in good thermal contact with the inserted ampoule. Registration of the power signal and calibration are similar to that for the flow microcalorimeter. The sensitivity of the recording system is 0.4 μ W, with baseline stability of the same magnitude over 24 h. Electrical calibration was cross-checked by a calibration reaction process as described by Chen and Wadsö (12). For all determinations of the power in a sample, water was used as non-reactive reference solution.

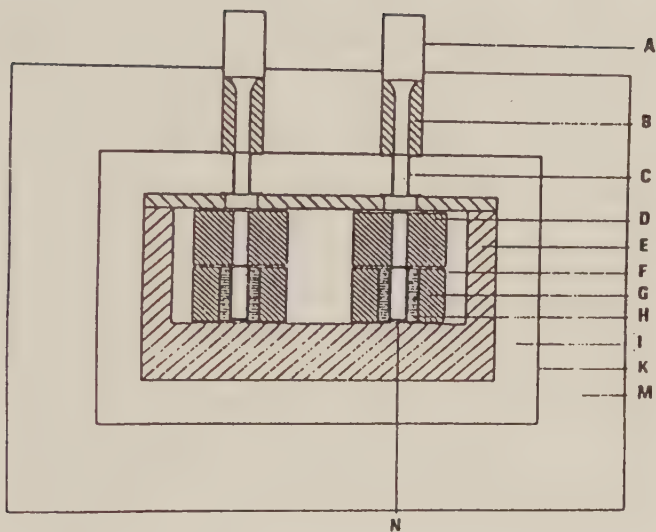


Fig. 2. The static ampoule microcalorimeter. The positions A, B, C and D are successive, resting positions to temperature equilibrate the ampoules. The thermopile H is in contact with the heat sink G, positioned in the main heat sink E. Air space F and I. Calorimeter jacket K, in a water thermostat M; ampoule holder N. Reprinted from (ref. 69).

The three-channel prototype of the multi-channel microcalorimeter - type C

Fig. 3 shows a multi-channel instrument of a construction nearly identical to type C. The basic components of the Type C microcalorimeter are in principle akin to those of the type B microcalorimeter, with a number of constructional refinements. The calorimeter holds three pairs of independent measuring units. Operational amplifiers are enclosed in water-tight steel tubes and placed in the thermostated water bath at 37°C . The electronic units are contained in an electric console from which electrical calibration powers in the range $5\ \mu\text{W} - 10\ \text{mW}$ can be applied. A precision thermostated water bath maintains the calorimeter temperature at e.g. (37 ± 0.01) and the bath can be set for temperatures in the range $(20 - 37)^{\circ}\text{C}$. The ampoules have 1.3 ml volume and are sealed by teflon discs of 0.5 mm thickness. The sensitivity is $< 0.1\ \mu\text{W}$ with a baseline stability of the same magnitude over 24 h.

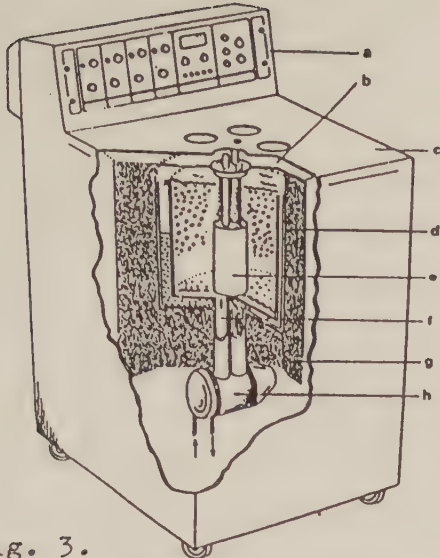
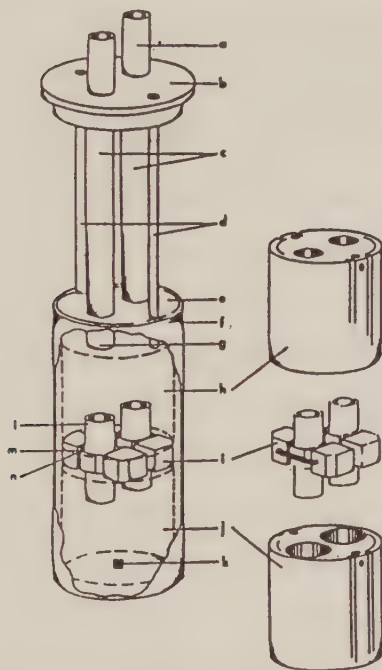
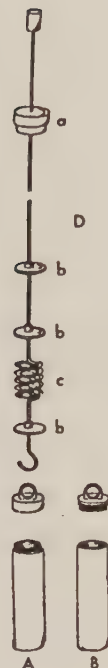


Fig. 3.

The multi-channel instrument: a, electric console; b, inner lid; c, outer lid; d, water bath; e, twin calorimeter; f, pump outlet tube; g, polyurethane foam insulation; h, centrifugal pump. Reprinted from (ref. 66).



Twin calorimetric channel used with cylindrical insertion vessels: components of the multi-channel microcalorimeter. a, Ryton connecting tubes; b, lid; c, 16 mm steel tubes; d, 6 mm steel tubes; e, steel lid; f, steel vessel; g, Ryton connecting tubes; h, aluminium block; i, small aluminium block; j, aluminium block; k, steel spring; l, ampoule holder; m, Peltier effect plate; n, calibration heater. Reprinted from (ref. 66).



Stainless steel ampoules A, B with teflon and O-ring seals, respectively. D is stylus for ampoule insertion. Reprinted from (ref. 66).

6. METHODOLOGY

6.1 Erythrocytes

The erythrocytes used in the studies (Papers I, II, III, IV) were obtained from whole blood free from other cell types. 20 to 40 ml of blood containing heparin, 14.3 USP/ml, were collected. Plasma and erythrocytes were immediately separated by centrifugation at 1000 g for 10 minutes. Cell-free plasma was obtained by recentrifugation at 8000 g for 15 minutes at 4 °C. The buffy coat of leucocytes was removed by gentle aspiration. The erythrocytes were washed twice with five volumes of either 0.9 g/l NaCl or choline Ringer buffer of composition: choline chloride 146 mM, $MgCl_2$ 1.0 mM, $CaCl_2$ 1 mM, NaCl 5 mM, orthophosphoric acid 2.5 mM, pH 7.40. In each washing procedure, remnants of the buffy coat and the upper layer of erythrocytes were removed by aspiration. About 20% of the erythrocytes were lost by this washing procedure. The cells were resuspended in plasma or buffer at hematocrits of about 10% or (38-50)%. The erythrocyte suspensions so obtained contained insignificant numbers of platelets and leucocytes.

Literature searches reveal that the majority of metabolic studies on erythrocytes were performed on cells freed from other cell types by the centrifugation method described above. These cells were found to metabolise glucose and produce adequate levels of ATP for the maintenance of the vital gradients of Na^+ and K^+ by active transport. However, in health, peripheral blood contains mature erythrocytes of different ages, about (0-120) days. Age-dependent metabolic changes in peripheral blood erythrocytes have been described. They

include changes in buoyancy, osmotic fragility and enzyme activity (61). In the methods described for age classification of erythrocytes by centrifugation, young cells which have low densities appear in the upper layer while the older, denser cells are packed in the lower layer (14). The young cells were shown to have higher glycolytic enzyme activity and ATP levels when compared with the older, denser cells (61, 62). Thus, by using the centrifugation method for separating erythrocytes from other cell types in the studies of Papers I, II, III, and IV, a fraction of the young cell population was lost during the preparation. The metabolism of the cells obtained by the centrifugation method may therefore not represent that of the physiological population. In an earlier calorimetric study, heat production rates in suspensions of erythrocytes purified by the centrifugation method and by a column elution method (57) were compared. In the latter method, whole blood was applied to a column consisting of a mixture of Sephadex G-10 from Pharmacia, and sulphoethyl cellulose, SE 50, from Serva. The erythrocytes were eluted from the column with buffer, while the leucocytes and platelets remained adhered to the column by ionic bonding. This simple procedure gave about 100% erythrocyte recovery and complete separation from the other blood cells. The elution method gave erythrocyte preparations with higher rates of heat production than preparations by the centrifugation method (47).

The centrifugation method was used in the studies of paper I and II before we were aware of the existence of the column elution method. However, we had to continue the use of the centrifugation method for the studies of papers III and IV, because attempts to use the column

method resulted in the binding of the erythrocytes firmly to the column when we used new batches of the sulphoethyl cellulose obtained from the same source as the material used by Monti et al. earlier (47). The new batches of sulphoethyl cellulose seemed to possess super-ionic binding properties for erythrocytes.

The column elution method, which is expected to give erythrocyte preparations more representative of the physiological population, should in general be the one of choice. This choice is particularly important when evaluating the metabolic status of erythrocytes for clinical application. Therefore, we continue to seek methods of decreasing the ionic binding between the column binding without losing the effectiveness for removing the other blood types and test other materials with favourable separation of erythrocytes. In this regard the separation method of Beutler et al. (7) is also being investigated. Renewed use of the column elution method coupled with new information on erythrocyte metabolism which has emerged from calorimetric studies (Paper III) would allow us to gain more insight into the mechanisms contributing to the high heat production rates in the different types of anemia reported earlier.

6.2 Lymphocytes

The separation of lymphocytes from other cell types is usually required for general metabolic studies (determination of respiratory and glycolytic rates) and for studies of various aspects of lymphocyte activity in the immune system. The widely used one-step isolation of lymphocytes from blood by the method of Böyum (11) has

been found to give lymphocyte preparations with monocyte contaminations. Soman et al. (64) found a range of (20-27)% monocyte contamination of their lymphocyte preparation when assessed by three different criteria and also found that the degree of contamination was dependent on the original monocyte count in whole blood.

Several methods of freeing lymphocyte preparations of phagocytic cells have been reported in recent years. All the methods exploit either the phagocytic or the adherence property of the cells. One type of approach was the use of nylon wool columns (16), rayon wool columns (28), glass bead columns (1), plastic or glass surface (38) and Sephadex G-10 beads (26) as adherent matrix. Jerrel et al. (26) found that both the use of Sephadex G-10 columns and carbonyl iron gave lymphocyte preparations with the same proportion of B and T subpopulations as in whole blood with optimum depletion of phagocytic cells. Similar results using carbonyl iron were obtained by Holm et al. (21).

The large intersubject variations in heat production rates in lymphocyte suspensions obtained by Górski et al. (17) and Bandmann et al. (5) indicated possible effects of phagocytic cell contamination in these preparations. Therefore, in the studies of Papers V, VI, and VII we applied the carbonyl iron method and tested the Sephadex G-10 method in Paper V for further purification of our lymphocytes prepared by the method of Böyum. These methods are described in Paper V and were found to be effective in removing phagocytic cells from lymphocyte preparations.

6.3 Plasma and buffer

Plasma was obtained from whole blood by centrifugation at 1000 x g for 10 minutes. This was followed by recentrifugation at 10000 x g for 15 minutes at 4 °C. In some experiments, where the high speed centrifuge was not available, recentrifugation was carried out at 3000 x g for 30 minutes at 25 °C. In general, plasma was used as suspension medium for both erythrocytes and lymphocytes except in paper III, where tris-HCL buffer was tested as suspension medium.

RPMI 1640 (54) containing Hepes, 20mM, and NaHCO_3 , 36 mM, was used as an alternative suspension medium for lymphocytes in Paper V. In many studies reported on lymphocyte cultures, this buffer system has been found to be non-toxic to the cell. However, the above concentration of NaHCO_3 was found empirically to be the one which gave steady state power-time curves and negligible "crowding effects" in the physiological range of cell concentration.

7. SOURCES OF ERROR

Two types of errors are expected to affect the values obtained for the power in blood cells. Random errors are responsible for variations in repeated calorimetric determinations and cell enumeration. This type of error can usually be estimated from repeated determinations and reduced by standardisation of experimental procedures. The other type of error, the systematic error, will reduce the accuracy of the determined power values. The error may

arise from the calorimetric determinations or from the preparative procedure of the blood cells. The heat observed in a calorimetric determination is the sum of the heat produced in the blood cell suspension (heat of metabolism) and the heat from other processes unrelated to metabolism, e.g. friction, evaporation and condensation. If the procedures for calorimetric determinations are not arranged to exclude these extraneous heat sources, large systematic errors can be included in the determined power values.

For erythrocytes and especially lymphocytes, incomplete separation from other cell types is a main source of systematic error, which can lead to large inter-donor variations, as may be seen in Paper V. Mechanical damage to some of the cells during preparation is likely to cause leakage of intra-cellular materials and produce cells with abnormal metabolism. Table I gives a summary of some calorimetric sources of error in power value determinations and the type of calorimetric technique where the sources of errors can be significant and problematic. After improvement and careful standardisation of the operational procedures of the static microcalorimeters (B, C), we believe that only item number 7, of all the sources of errors listed in Table 1, viz cell sedimentation, may adversely affect accuracy of the power values reported for erythrocytes and lymphocytes.

Table 1. Some calorimetric sources of error in determinations of power values in blood cells.

Sources of error	Type of Technique ^a
1. Baseline instability and poor reproducibility	Flow (A)
2. Poor definition of steady state	Flow (A)
3. Inadequate sample delivery to reaction vessel (charging)	Flow (A)
4. Evaporation and condensation	Static (B, C)
5. Heat of friction	Flow (A)
6. Contamination of reaction vessel	Flow (A)
7. Cell sedimentation	Flow, static (A, B, C)
8. Adhesion of cells and proteins to walls of reaction vessel	Flow (A, B, C)
9. Calibration	Flow (A)

^a Type of calorimetric technique where the error sources can be significant

In this study, the errors connected with the method of cell preparation have been reduced by using mild isolation procedures and by using cell concentrations in the range where the "crowding effect" appears to be insignificant.

8. RESULTS AND COMMENTS

8.1 Erythrocytes

8.1.1 Heat production by human erythrocytes in vitro: a study of two different populations.

With careful standardisation of the procedures of cell preparation and flow calorimetric measurements, we obtained a mean heat effect (power) of (100 ± 13) mW/l erythrocyte from 50 male Nigerian blood donors. This value is lower than the mean value of (115 ± 19) mW/l cells obtained in the study of Levin & Boyo (31) for erythrocytes from 50 Swedish blood donors using the same type of flow microcalorimeter and experimental procedures. We were at first tempted to relate the difference in the power to the biological and geographical differences of the two populations. Determination of power values in erythrocytes from 25 Swedes visiting Nigeria gave a mean value of (99 ± 7) mW/l cells. This mean value was in agreement with the mean value obtained for the 50 Nigerians. Comparison of these mean values and the mean value of (80 ± 6) mW/l obtained by Monti & Wadsö (45) using static (zero flow) microcalorimetry suggested that the difference between our mean values and that of Levin (31) most probably arose from differences in flow rate. We therefore concluded that heat production by human erythrocytes in health is constant and independent of the biological and the geographical differences of the two populations. The results of this study show the importance of standardisation of experimental procedures and definition of experimental conditions in flow microcalorimetry.

8.1.2 The effect of cellular flow and viscosity on heat production measured by flow reaction calorimetry by human erythrocytes in vitro.

This paper draws further attention to the influence of the flow rate of erythrocyte suspensions in plasma on determined heat effects (power) when using the flow microcalorimetric technique. The use of a peristaltic pump for introducing the blood cells to the reaction vessel of the calorimeter often caused shifts in the reference baseline and increase in power as the flow rate increased in the range (14 to 35) ml/h. At flow rates higher than 35 ml/h, the thermograms (power-time curves) continued to increase, resulting in poor and variable steady state curves. The power varied directly with flow rates in the range (14 to 35) ml/h. The linear relationship gave an intercept value of (72 ± 9) mW/l erythrocytes at zero flow rate. The mean intercept value obtained from 10 experiments was comparable to the value of (80 ± 6) mW/l erythrocytes obtained by Monti & Wadsö (45), using a static type of microcalorimeter in which the flow rate may be assumed to be equal to zero. When the erythrocytes were stored at 37 °C for 72 hours to deplete substrates, the heat production rate at a flow rate of 25 ml/h was 58 mW/l cells while the intercept value approached zero mW/l cells. The increase in viscosity known to accompany energy depletion during aging is likely to increase the friction between the erythrocyte suspensions and the walls of the calorimeter vessel in flow microcalorimetry and cause a subsequent increase in the determined power. That friction was the probable source of this persisting heat was confirmed later by monitoring heat production rates under identical conditions using the static

technique. After aging of the cells for 72 hours, heat production rates were practically zero.

The general increase in heat production rates with flow rate could have a metabolic origin. Increases in the pH and the degree of oxygenation were observed in all the samples at high flow rates after calorimetry. It is concluded that, although the flow microcalorimetric technique is simple and rapid, these flow-dependent metabolic and biophysical properties of erythrocyte suspensions are likely to introduce large systematic errors in determined power values. In this respect, the static microcalorimetric technique seems superior.

8.1.3 The relationship between heat production rate, glycolysis and Na^+ , K^+ -ATPase activity in human erythrocytes.

The two enzyme systems, enolase and Na^+ , K^+ -ATPase, are involved in ATP production and utilisation in the erythrocyte. The effect of inhibiting these two enzymes on the power observed in erythrocyte suspensions was evaluated. Inhibition of the Na^+ , K^+ -ATPase activity induced mean decreases of power (12 ± 2) mW/l erythrocytes and (14 ± 5) mW/l erythrocytes in tris-HCl and plasma respectively. The decreases in power values are in accordance with the known effect of Na^+ , K^+ -ATPase inhibition on the rate of glycolysis (4, 59).

Inhibition of lactate production by enolase was accompanied by a mean decrease in power to 46% of its initial value. There was a

linear correlation between the total power and the power associated with lactate production. The values 39 mW/l cells and 44 mW/l cells obtained for the power associated with lactate production by calculation and by enzyme inhibition respectively are similar. The calculated power values in excess of the values expected for lactate production from glucose under steady state rate of glycolysis are 38 mW/l by calculation and by enzyme inhibition. However, by calculation, a power of 23 mW/l remains unaccounted for after due consideration of the power contribution from lactate production and activity of the pentose phosphate pathway. The probable degradation of 2,3-DPG (59) may be implicated in the production of this residual power.

From determined mean rate of glucose consumption, an estimate of 19% of the total power was found for the activity of the pentose phosphate pathway (PPP). This calculation was based on the findings of Murphy (55) that only 11% of the total glucose consumed is metabolised to carbon dioxide in the PPP.

These results indicate that specific enzyme inhibition could be used to monitor various enzyme contributions to observed rates of heat production in erythrocytes.

8.1.4 Erythrocyte heat production in human obesity.

Microcalorimetric investigation of the sodium potassium pump and cell metabolism

The activity of the Na^+ , K^+ -ATPase is the enzymatic expression of

the so-called Na^+ , K^+ -pump, the mechanism by which the erythrocyte maintains constant concentrations of intracellular Na^+ and K^+ with the energy derived from ATP. The cardiotonic steroid ouabain is a known specific inhibitor of the membrane enzyme, the Na^+ , K^+ -ATPase, see e.g. (72). Much effort has been directed to the investigation of the significance of the pump in the pathogenesis of human obesity. In comparison to erythrocytes from normal subjects, reduced, increased and normal Na^+ , K^+ -pump activity have been reported in human obesity. These findings were based on two aspects of the Na^+ , K^+ -pump characteristics, viz the number and availability of binding sites for ouabain at the cell membrane and the activity of the Na^+ , K^+ -ATPase. In this study, we investigated another aspect of the Na^+ , K^+ -pump. We monitored the response of the over-all rate of metabolism (quantified in power units) to inhibition of the Na^+ , K^+ -ATPase activity in erythrocyte suspensions in plasma. This response expresses the relationship between the processes of ATP production and utilisation and hence the energy balance in the cell.

The mean values of total heat production rates were (107 ± 13) mW/l cells, $n = 33$, in the control group and (118 ± 17) mW/l, $n = 25$, in the obese group. The difference between the two groups is significant ($p < 0.05$). However, there was no correlation between the total power and body weight. Although we cannot explain the increased total power values in the obese group, the finding is consistent with reports of elevated basal rates in obese subjects as estimated by indirect calorimetry (25) and by direct calorimetry (23). Specific inhibition induced the same decrease of heat production rates in the

two groups (14 ± 5) mW/l cells, $n = 33$, for the normal subjects and (13 ± 5) mW/l cells, $n = 25$, for the obese subjects. In neither group was there a correlation between the ouabain-inhibitable power and body weight. Our findings speak against the hypothesis that a metabolic derangement secondary to defective Na^+ , K^+ -pump activity is of importance in the pathogenesis of human obesity.

8.2 Lymphocytes

8.2.1 Heat production in human blood lymphocytes. A methodological study

Heat production rates (power) in peripheral blood lymphocytes from healthy subjects were determined under some defined experimental conditions. This kind of methodological study is required for characterising the thermochemical properties of lymphocytes at the molecular level, for studies of lymphocyte metabolism in general and for studies of the immune functions of the lymphocyte by microcalorimetry. The power values obtained for suspensions of lymphocytes were found to be dependent on the following parameters: type of anticoagulant used for whole blood, the presence of other blood cell types in the lymphocyte preparation, time of storage at 25°C , type and pH of suspension medium and temperature of calorimetric measurement.

The mean power value, P , for lymphocytes prepared from heparinised blood and suspended in plasma was (1.8 ± 0.3) pW/cell and (2.7 ± 0.5) pW/cell for lymphocytes prepared from defibrinated blood.

The reason for the significant difference ($p < 0.005$) is not clear. The use of heparin as anticoagulant was found to be simple and to give reproductive power and power-time curves. It was therefore the preferred anticoagulant for further studies.

The most widely used isolation method for lymphocytes, the method of Böyum (11), gave lymphocyte preparations with (2 to 37) % phagocytic cells. Such preparations gave decreasing power-time curves and poor reproducibility of power values. Removal of the phagocytic cells in the lymphocyte preparations resulted in steady state power-time curves and reproducible power values.

The power value for lymphocyte suspensions in plasma decreased (5.0 ± 0.3) % per hour when stored at room temperature. The decrease was found to be due to the increase in pH of the plasma suspension medium during storage. This undesirable effect could be avoided by storage of the plasma at 4 °C during cell preparation and resuspension of the cells in the plasma just before microcalorimetry.

A linear pH dependence of power values was found in the pH range 7.20 to 7.50. From this a general relationship given by

$$P_{\text{exp}} = P^0 (1 + 0.78 \Delta\text{pH}) \quad (1)$$

was found, where

P_{exp} is experimental pH

P^0 is power at pH 7.40

ΔpH is increase in pH above 7.40

By using the power value at 37 °C as reference, a linear relationship was found between the relative power values and the reciprocal temperatures in the range 25 °C to 37 °C. From the linear Arrhenius plot, an apparent activation energy of 62.9 kJ/mol was obtained. Such linear relationships and activation energies of 83 kJ/mol and 52 kJ/mol have been obtained in earlier studies for erythrocytes (46) and platelets (49), respectively, by similar calorimetric techniques.

In the concentration range $(2.0 \text{ to } 6.0) \times 10^6$ cells/ml, power values (heat production rates per lymphocyte) were found to be independent of cell concentration. For pair-wise determinations of P values at high and low cell concentration, a spread of about 10% of the mean was obtained.

The P^0 values for lymphocytes suspended in plasma and RPMI 1640 containing NaHCO_3 was (1.7 ± 0.2) pW/cell, $n = 4$, and (1.6 ± 0.2) pW/cell, $n = 6$ respectively. These values are similar and suggest that the medium RPMI 1640 containing NaHCO_3 as described may be a good alternative suspension medium for lymphocytes in health.

8.2.2 Heat production in lymphocytes in chronic lymphocytic leukemia

The mean heat production rate, P , in a group of healthy subjects was (2.6 ± 0.5) pW/cell, $n = 8$, and (1.8 ± 0.5) pW/cell, $n = 10$, in a group of patients with chronic lymphocytic leukemia (CLL). The

difference in P values is significant, $P < 0.005$. The lower rate of heat production per lymphocyte in CLL may represent a decrease in over-all metabolic activity of the cells. Decreased activity of the pentose phosphate pathway in lymphocytes of CLL has been reported by Brody (10).

Using the normal peripheral lymphocyte counts, calculation of the total heat output expected from the intravascular lymphocyte pools gave the values 0.03 W for the healthy subjects and 1.80 W for the patients with CLL. This increased heat output together with that expected from the larger pool of lymphocytes in lymphoid tissues in CLL may explain some of the symptoms of the hypermetabolic state often seen in CLL.

Further studies based on the findings of Brody and the decreased power obtained in this study for lymphocytes of patients with CLL might yield some biochemical information of prognostic value in CLL.

8.2.3 Metabolic activity of lymphoma cells and clinical course in non-Hodgkin lymphoma (NHL)

Heat production rates, P, in plasma suspensions of malignant cells from 21 patients with non-Hodgkin lymphoma were determined. The mean P value for tumor cells from the patients who responded to therapy (responders) was (3.1 ± 0.8) pW/cell, $n = 11$. The corresponding value for patients who had progressive disease (non-responders) was (5.5 ± 3.3) pW/cell, $n = 10$. The same trend in P

values was obtained for the peripheral blood lymphocytes, (2.4 ± 0.5) pW/cell and (3.1 ± 1.1) pW/cell for responders and non-responders, respectively. The mean P value for the responders was similar to the mean value of (2.7 ± 0.7) pW/cell obtained for peripheral blood lymphocytes from normal subjects. The results suggest that high heat production rates in lymphoma cells are associated with aggressive growth of the tumor and with poor prognosis.

Recently, density distribution patterns of cells obtained from tumor biopsies in NHL were found to be of prognostic value (22). Lymphoma cells of low density showed unfavourable histology and high spontaneous tumor cell proliferation, while those of high density were associated with favourable histologic features and low proliferation.

Recently, Hagberg et al (19) have proposed the use of serum lactic dehydrogenase, S-LDH, for prognosis in NHL. They found elevated S-LDH in patients with NHL. These levels were found to be significantly correlated with the spread and grade of malignancy of the disease. This aspect of the tumor metabolism is one that is amenable to calorimetric studies at the molecular level.

9. GENERAL OBSERVATIONS AND CONCLUSION

The tendency in the last years has been towards the development of sensitive calorimeters capable of detecting precisely the power in cell suspensions of low concentrations. The power in a suspension containing 1.0×10^6 lymphocytes is about 2 μ W. Accurate determination of such low power puts stringent demands on the

standardization of experimental procedures. Heat contributions from processes unrelated to the cell's metabolism must be minimal. Examples of such contributions are discussed under "Sources of Errors", page 26. Two other sources of error will cause variable baselines and prolong the approach to steady state in calorimetric determinations. The first concerns the humidity in the calorimeter measuring cell. Fast and reproducible response of the thermopile heat sensors requires low and identical humidity around the thermopiles of the reference and reaction cells. Therefore calorimetric determinations should be conducted in carefully dried ampoules with perfect seals.

The second source of error concerns the sealing of the ampoules. In earlier ampoule models, rubber O-rings were used as seals and found suitable. But at the high sensitivity of the new type of microcalorimeters, a slow approach to steady state was observed with rubber O-rings as seals, leading to distortion of power-time curves. This effect is probably due to a slow relaxation rate of the rubber after the torsion and compression of sealing. The effect could be reduced by light greasing of the O-ring before sealing. It was possible to cancel this effect completely by simultaneous charging and sealing of both the reference and reaction ampoules. In the prototype of the LKB Bio-Activity Monitor, BAM, the apparent relaxation effect was avoided by the use of flat teflon discs as ampoule seals.

The static microcalorimetric method has some advantages over the flow method. The former requires about a tenth of the quantity of the

sample required in the flow method. Sample size definition and introduction to the reaction vessel are more precise. However, due to sedimentation, a gradient of cell concentration develops and crowding effects, e.g. density dependent inhibition of O_2 -consumption of the type described by Hedekov et al. (20), may occur. For erythrocytes this does not seem to be a problem, but for lymphocytes a deficiency of oxygen would limit respiration. The P values obtained for lymphocytes in the range $(1-5) \times 10^6$ cells seemed to be independent of oxygen content of the sample, since the power per cell varied within about 10% of the mean of pair-wise determinations at two different concentrations. However, in RPMI buffer, P values were found to be strongly pH dependent (5% of P^O for every 0.01 change in pH). In this buffer and in phosphate buffer, the crowding effect was very pronounced in the range $(2.0 \text{ to } 6.0) \times 10^6$ cells/ml. This crowding effect could be completely eliminated by addition of $NaHCO_3$ at about 36 mM to the buffer (pH 7.30) before addition of the lymphocytes. In the buffer containing $NaHCO_3$, the pH dependence of P values of lymphocytes was similar to that of plasma suspensions, 0.8% of P^O for every 0.01 change of pH. The biochemical basis of this favourable effect of bicarbonate is not clear, although bicarbonate and carbon dioxide have long been known to be essential for human cells in vitro (15, 16).

The standard procedure for calorimetric measurements consisted of using 1.0 ml water as a non-reactive reference for all determinations. For erythrocytes and lymphocytes suspended in plasma or buffer, the power in the suspension medium was determined in a separate experiment

and subtracted from the total power for the suspension to obtain the power for the blood cells. In a set of four experiments conducted using a prototype of the multi-channel microcalorimeter, the reference ampoule was charged with plasma and used simultaneously as reference for lymphocytes suspended in plasma. This way the plasma power was directly subtracted during the experiment. The mean power per lymphocyte obtained by this procedure varied within 2% of that obtained by the standard procedure. Therefore this alternative procedure may be used, especially when time is a limiting factor.

In conclusion, the overall information derived from the studies presented here indicates that static ampoule microcalorimetry is suitable for assessment of the metabolism of erythrocytes and lymphocytes and may be of use as a complement to existing methods in differential diagnosis of lymphoid diseases. For further studies on blood cells, the following suggestions are recommended for obtaining precise power values that may be correlated validly with related biochemical parameters, e.g. rates of O_2 consumption or lactate production and of ATP utilisation.

- i) Improvement of cell counts to match the precision of calorimetric determinations.
- ii) Use of a cell-suspension system that will prevent sedimentation, e.g. by applying mild stirring of the cell suspension during calorimetric determination.
- iii) Development of experimental procedures for specific reactions of individual blood cells using whole blood systems, e.g. simultaneous determination of power values and

biochemical correlates during phagocytosis by neutrophils and monocytes in the presence of the other cell types. In this connection, specific enzyme inhibition is likely to give valuable information on the thermodynamics of partial reaction steps of the metabolic pathways.

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HEAT PRODUCTION BY HUMAN ERYTHROCYTES IN VITRO: A STUDY OF TWO
DIFFERENT POPULATIONS

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In an earlier study using the LKB flow microcalorimeter for investigating human erythrocyte metabolism we obtained a heat effect of 86 ± 11 (SD) calories per hour per litre of erythrocytes from 50 Nigerian male blood donors (1). Erythrocytes from a comparable Swedish group of blood donors with the same hemoglobin genotype investigated by Levin and Boyo gave a value of 99 ± 16 calories per hour per litre of cells (2). However Monti and Wadsö using an ampoule drop calorimeter in which the cells were undisturbed obtained 69 ± 5 calories per hour per litre of erythrocytes (3), a value different from Levin and Boyo's value for a comparable Swedish population. In this paper, the heat effect of erythrocytes from Swedish male donors visiting Lagos, Nigeria was measured under identical conditions as cells from Nigerian male donors in Lagos in an attempt to establish a significant difference, if any, between these two biologically and geographically different populations and also test our methodology and instrumentation using a non-Nigerian population.

MATERIALS AND METHODS. 20 ml of venous blood was collected aseptically from healthy male donors into heparinised bottles (20 i.u. heparin/ml blood). The mean hemoglobin concentration was 132 ± 10 g/l and 136 ± 8 g/l for the Nigerian and Swedish donors respectively. Haematological analyses were done by the usual standard methods (4). Preparation of the erythrocytes, instrument specifications, heat effect measurements and calculations were as described earlier (5).

RESULTS AND DISCUSSION. As can be seen in the table, the values for the heat effects obtained for the Nigerian and Swedish male donors in this study using identical experimental conditions, flow rates and

same type of calorimeter are in good agreement. The value 99 ± 16 cal/h/l cells obtained by Levin and Boyo using the same type of calorimeter but a different flow rate, is apparently different from the value obtained in this study for a comparable group of male donors. These results are in accord with our earlier finding that the total heat effect increases with the flow rate of the erythrocytes through the calorimeter within the range 14 ml/h to 35 ml/h (6).

These results illustrate the importance of using similar and well defined experimental conditions for comparative calorimetric studies of cells from similar and different human populations. The similarity in values obtained for Nigerians and Swedes in this study confirms our earlier assertion that heat production by human erythrocytes is constant in health and can be used as a reliable index of metabolism (5).

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Heat effect versus flow rate

	Heat effect cal/h/l	Flow rate ml/h	n	Type of Calorimeter
Present study				
Nigerians in Nigeria	86 ± 11	25	50	Flow
Swedes in Nigeria	85 ± 6	25	25	Flow
Swedes in Sweden (Levin & Boyo)	99 ± 16	35	50	Flow
Swedes in Sweden (Monti & Wadsö)	69 ± 5	—	28	Ampoule Drop

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THE EFFECT OF CELLULAR FLOW AND VISCOSITY ON HEAT PRODUCTION BY HUMAN
ERYTHROCYTES MEASURED IN VITRO BY FLOW REACTION CALORIMETRY

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Monk and Wadsö (1), while testing the flow reaction calorimeter showed in their "zero effect" experiments that the heat effect associated with the flow of water through the calorimeter changes very rapidly with the flow rate and viscosity and may lead to large systematic errors if the heat effect of the process under study is small. In the LKB flow microcalorimeter adapted for the study of erythrocyte metabolism (2), a varioperpex pump was used for pushing the blood under investigation through the 18 carat gold tubing (1.0 mm cross section) of the calorimeter cell. The use of the pump was found to introduce an extraneous heat effect which could be cancelled out by the use of the zero suppressor circuit of a Keithley microvoltmeter used for monitoring heat changes in the calorimeter cell. The entry of blood with a viscosity different from that of the saline used for establishing an arbitrary base line was observed to introduce a change in flow rate of sample through the calorimeter and a corresponding change in base line. The following experiment was designed to investigate the heat effect associated with the flow properties of blood.

MATERIAL AND METHODS

From 20 ml of heparinised venous blood (20 i.u. heparin/ml blood) erythrocytes free from other blood cells were prepared as previously described (3). The erythrocytes were suspended in autologous plasma to obtain a cell suspension of hematocrit 40 ± 2 per cent. The cell suspension was then passed into the calorimeter by the use of an LKB varioperpex pump with an adjustable flow rate range from 0 to 100 ml/h. The flow rate of sample through the calorimeter was

changed by adjustment of the variperpex pump and the apparent flow rate was measured by noting the time taken for collection of 1 ml efflux from the calorimeter.

For each of the flow rates at which calorimetric measurements were made steady state baseline was established with physiological saline (9.0 g/l sodium chloride in water) flowing through the calorimeter. The red cell suspension was then introduced with a minimal column of air between the saline and the cell suspension. At steady state (as shown by a smooth thermogram plateau), the cell suspension was washed out of the calorimeter cell using saline. Calorimetric measurements and calibrations were carried out at flow rates of 14 ml/h, 25 ml/h, 35 ml/h.

RESULTS AND DISCUSSION

The flow rates 14 ml/h to 35 ml/h chosen for this study gave good non-changing steady state thermograms and are recommended for calorimetric measurements. Above a flow rate of 35 ml/h increasing thermogram plateaux were obtained and estimation of steady state end point was difficult. Below 14 ml/h the thermogram plateaux were steady over a reasonable period of about 5 minutes but the duration of a complete experiment was longer than desirable.

The table shows the variation of heat effects with apparent flow rates of blood through the calorimeter obtained from erythrocytes from ten different blood donors. As shown in the figure the heat effect extrapolated to zero flow rate, denoted H_0 , was 259 ± 32 Joules/h/l erythrocytes and corresponds to 62 ± 8 calories/h/l erythrocytes.

This value is comparable to the value of 69 ± 5 calories/h/l obtained by Monti and Wadsö using an ampoule drop calorimeter in which the cells were undisturbed (4). It seems reasonable therefore to assume that the H_0 value obtained in this study is a closer approximation to the heat effect due to metabolism of human erythrocytes.

The results also show that as much as 47 ± 4 percent of the total heat effect observed at 25 ml/h flow rate may be associated with the flow properties of the blood sample. These rather high and increasing heat effects obtained at increasing flow rates put a limitation on the sensitivity of the flow technique for measuring the heat of metabolism in erythrocytes. However, for more accurate determination of heat effects due to metabolism, the experimental design described in this paper minimises the errors introduced by differences in viscosity between saline and the blood samples. The methodology also makes possible the simultaneous measurement of changes in metabolism and flow properties of blood. During incubation at 37°C for up to 72 hours, the extrapolated heat effect, H_0 , declined progressively and approached zero. As much as 50 calories/h/l cells were observed to persist at a flow rate of 25 ml/h. It seems reasonable therefore to associate this large persisting heat effect mainly with increased viscosity subsequent to metabolic changes known to render blood cells more viscous.

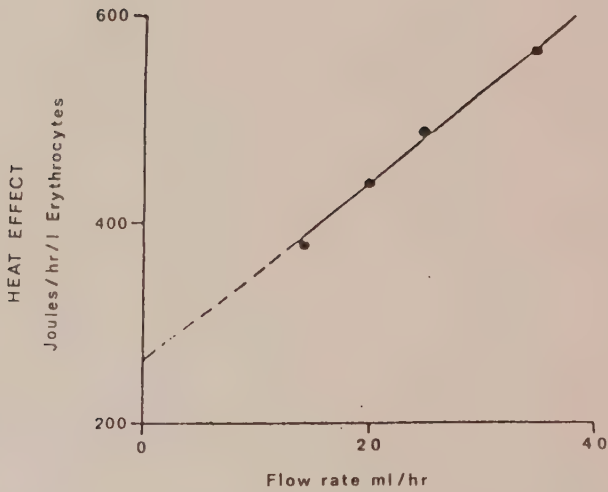
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TABLE 1

	Measured Heat Effects at Different Flow Rates Joules/hr/l cells				Heat Effects at Zero Flow Rates Joules/hr/l cells	$(H_{25} - H_0)$ Joules/hr/l cells	$\left(\frac{H_{25} - H_0}{H_{25}} \right) \times 100$
	H_{14}	H_{20}	H_{25}	H_{35}			
1	400	440	490	570	280	210	43
2	400	440	490	560	280	210	43
3	440	500	550	640	320	230	42
4	330	380	420	500	230	190	45
5	410	470	505	590	290	210	43
6	360	420	470	560	240	230	49
7	380	460	510	620	230	280	55
8	360	480	540	650	260	280	52
9	350	400	440	540	230	210	48
10	340	380	430	500	230	200	49
Mean $\pm$ S.D.	377 $\pm$ 40	437 $\pm$ 42	485 $\pm$ 46	573 $\pm$ 53	259 $\pm$ 32	245 $\pm$ 38	47 $\pm$ 4



Heat effect as a function of flow rate. Each point represents a mean value for 10 experiments (H_0) = 260 Joules/h/l cells.

THE RELATIONSHIP BETWEEN HEAT PRODUCTION RATE, GLYCOLYSIS AND
 Na^+ , K^+ - ATPASE ACTIVITY IN HUMAN ERYTHROCYTES

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SUMMARY

By microcalorimetry, a quantitative relationship between the over-all rate of heat production (power), lactate production and transmembrane activity of the Na^+ , K^+ - ATPase in human erythrocytes was evaluated. Specific inhibition of the Na^+ , K^+ - ATPase was accompanied by a reduction in the rate of heat production. Termination of lactate production by the inhibitory effect of sodium fluoride on the glycolytic enzyme enolase resulted in reduction of the heat production to 46% of its initial rate. The total power and the power associated with lactate production were positively correlated. The probable sources of the total thermal power in relation to known reactions of the metabolic pathways of the erythrocyte are considered.

INTRODUCTION

Microcalorimetry has been shown to be a sensitive technique for monitoring the metabolic activity in whole blood (3, 5, 10). The technique has also been used to investigate the metabolism of erythrocyte and other blood cells in health and in some pathological conditions (see e.g. 8, 16, 18, 21, 22). In comparison with erythrocytes from healthy subjects, increased rate of heat production by erythrocytes has been reported in different types of anemia (6, 14, 19), in liver disease (22), in uremia (23), and in hyperthyroidism (20). Although the biochemical basis of the increase in power is not known, the increase in population of young erythrocytes known to occur in some of the conditions is thought to be contributory (14). To understand the mechanisms behind the high rates, knowledge of the relative contributions from metabolic reactions of the glycolytic and hexose monophosphate shunt, the two known metabolic pathways of the erythrocyte, is necessary.

Earlier calorimetric studies of erythrocyte metabolism indicated a poor correlation between the rates of glucose consumption and heat production (16, 18). Minakami et al. (18), however, found a good correlation between the rates of lactate production and heat production by flow calorimetry. They also demonstrated the inhibitory action of sodium fluoride and iodoacetate, known inhibitors of glycolysis, on the rate at which erythrocytes produce heat. In a set of experiments, Levin (15) found that sodium fluoride caused a mean decrease of 44% in heat production rate in erythrocyte suspension in a

buffer medium.

In this study, the relationship between the thermal power and the process of lactate production in erythrocyte suspensions was investigated by inhibition of the glycolytic enzyme, enolase phosphopyruvatehydratase, (E.C.4.2.1.11). Sodium fluoride (NaF) was chosen for this study because its inhibition of enolase is specific and relatively rapid in terminating the production of lactate and 2,3-diphospho-glycerate (2,3-DPG) degradation without accumulation of pyruvate (18). By using NaF at a concentration which gave maximum reduction in power, the fraction of the total thermal power in plasma suspensions of erythrocytes associated with lactate production was determined.

The link between the transport of Na^+ , K^+ across the erythrocyte membrane and the energy of ATP derived from glycolysis is well established (26, 28). The hydrolysis of one mole of ATP is known to be coupled to the transport of 3Na^+ and 2K^+ across the membrane against their respective electrochemical gradients (35). In this study the effect of specific inhibition of the Na^+ , K^+ -ATPase (E.C.3.6.1.3) activity on the heat production rate in erythrocyte suspensions in plasma and tris-HCl buffer was evaluated.

MATERIALS AND METHODS

Preparation of erythrocyte suspensions. 20 to 40 ml of blood from healthy subjects was collected using heparin, 14.3 USP/ml blood, as anticoagulant. Plasma and erythrocytes were immediately separated by

centrifugation at 1000 g for 10 minutes. Cell-free plasma was obtained by recentrifugation at 8000 g for 15 minutes at 4 °C. The buffy coat of leukocytes above the erythrocytes was removed by gentle aspiration. The erythrocytes were washed twice, each time with five volumes of choline Ringer buffer of composition: choline chloride 146 mM, $MgCl_2$ 1.0 mM, $CaCl_2$ 1 mM, NaCl 5 mM, orthophosphoric acid 2.5 mM, pH 7.40. In each washing procedure, remnants of the buffy coat and the upper layer of erythrocytes were removed by aspiration.

In one set of experiments, the erythrocytes were resuspended in cell-free plasma at hematocrits of (40-50) % close to the hematocrit of the original whole blood. In another set of experiments, the erythrocytes were suspended at hematocrit of about 10% in tris-HCl buffer of composition: NaCl 150 mM, KCl 3 mM, $CaCl_2$ 2 mM, $MgCl_2$ 1 mM, NaH_2PO_4 0.4 mM and glucose 5 mM, pH 7.30. This buffer system was that used by Ataullakhanov et al. (2) in their experimental verification of a quantitative model of erythrocyte glycolysis and dependence on Na^+ , K^+ - ATPase activity.

Calorimetric measurements

Two types of heat conduction microcalorimeters were used. The first is a prototype of the LKB Bio Activity Monitor (BAM) described recently (32). This is a heat-conduction microcalorimeter with four channels and sensitivity of 0.05 μW . The second type consisted of two nearly identical calorimeters (33) of the kind used in earlier blood cell experiments (3). Heat production rates (power) in

erythrocyte suspensions were determined under static conditions in 1.1 ml or 1.3 ml stainless steel ampoules as previously described (19). Heat production rates were recorded over a period of one to three hours until power-time curves (heat production profile) of zero slope indicated steady states of at least 30 minutes' duration.

Inhibition experiments

As inhibitor of Na^+ , K^+ - ATPase activity, a 15 mM stock solution of ouabain (Strophanthin G from Sigma) in 0.9 g/l NaCl was used. In four preliminary experiments, 10 to 50 μl of stock ouabain solution was added to 1.2 ml of erythrocyte suspension in plasma or buffer, and gently mixed. 1.0 ml of the mixture was used for calorimetric determinations. Similar aliquots of the same erythrocyte suspension, containing 0.9 g/l NaCl instead of ouabain, were added to control samples. The concentration of ouabain in the erythrocyte suspension which gave maximum inhibition of metabolic rates was determined and used in subsequent experiments. Intracellular concentrations of Na^+ and K^+ in the erythrocytes were determined before and after calorimetry by a method described by Bengtsson *et al.* (4), using a Unicam emission flame photometer, SP 90.

For inhibition of lactate production, a 1 M stock solution of NaF (Sigma) in 0.9 g/l NaCl was used. In each of a set of four experiments with erythrocytes from different donors, 12 to 48 μl of stock NaF solution was added to 1.2 ml portions of erythrocyte suspension in plasma. 1.0 ml of the mixture was used for determination of heat production rates. The concentration of NaF

which gave maximum decrease in power values was used in subsequent experiments.

Glucose consumption

The rates of glucose consumption and heat production were simultaneously determined in plasma suspensions of erythrocytes from four different donors. Glucose concentrations were determined in duplicates by the glucose oxidase method of Werner et al. (34), using a Boehringer Diagnostic kit and enclosed analytical procedures. The rate of glucose consumption was determined from samples taken during the 40 minutes when steady state power values were observed.

RESULTS

Power-time curves

Plasma suspensions. Heat production rates recorded over a time interval are referred to as power-time curves. The type of power-time curve obtained for erythrocytes was dependent on the suspension medium. For plasma suspensions, steady state power-time curves of the type shown in Figure 1 were obtained after the samples were introduced into the calorimeter. This steady state lasted for some 30 minutes and is denoted steady state I to distinguish it from the steady state obtained with erythrocytes in Tris-HCl buffer medium. Thereafter, the power continuously decreased for several hours and failed to reach steady state. For plasma suspensions containing ouabain, power-time curves were similar in form to those of the control samples, but their P values were lower than those of the control samples. For similar suspensions containing NaF, power-time curves of the type shown in

Figure 2 were obtained. These curves had initial peaks, then decreased to steady state values in about two hours.

Suspensions in tris-HCl buffer. For suspension of erythrocytes in tris-HCl buffer with and without ouabain, initial power peaks of approximately the same magnitude as the plasma steady state power value were obtained 30 minutes after sample introduction into the calorimeter. This was followed by a rapid decline to steady state power values in about two hours. In contrast to plasma suspensions, buffer suspensions reached steady states after about 2.5 hours. These steady states lasted for three to four hours and are denoted steady state 2. At this steady state the power value for the sample with ouabain was lower than the power value for the corresponding sample without ouabain.

Power values and glucose consumption rates.

The P values from four experiments were (77 ± 3) mW/l erythrocytes, pH 7.35. The corresponding rate, V_g , for glucose consumption was $(0.270 \pm 0.008) \times 10^{-6} \text{ mol s}^{-1} \text{ l}^{-1}$.

Inhibition of metabolism

Lactate production. Table 1 shows the data for simultaneous determinations of power values in erythrocytes suspended in plasma with and without fluoride. There was marked reduction of steady state power values and slight shifts to higher pH for samples containing NaF when compared with controls. The data in Table 1 was obtained from erythrocyte suspensions in plasma from 9 healthy sub-

jects and two patients. The mean fluoride inhibitable rate, ΔP , for the healthy subjects was (44 ± 8) mW/l erythrocytes. The corresponding values for a patient with liver disease and one with chronic uremia were 70 mW/l and 92 mW/l, respectively. Although lactate production was complete (18), erythrocytes in plasma continued to produce heat at a mean residual steady rate of (38 ± 5) mW/l. This residual rate was equivalent to (46 ± 4) % of initial rates of heat production in the absence of inhibitor. As shown in Figure 3, there is a good correlation between the total power P and fluoride inhibitable power, ΔP .

Na^+, K^+ -ATPase activity. In the presence of ouabain, reduction in power values occurred in both plasma suspensions and tris buffer suspensions of erythrocytes. For plasma suspensions of erythrocytes from 9 donors, the concentration of intracellular Na^+ determined at the end of calorimetry increased by (2.8 ± 0.6) mmol/l cell H_2O in the presence of ouabain. This increase represents (36 ± 8) % of the intracellular Na^+ concentration in the absence of ouabain. The corresponding changes in intracellular K^+ concentration were low and within the error limits of the determinations. The change in intracellular Na^+ concentration was therefore taken as a better indicator of ouabain inhibition of Na^+, K^+ -ATPase. The ouabain inhibitable power was (12 ± 2) mW/l erythrocytes, $n = 7$, in tris-HCl buffer suspensions and (14 ± 5) mW/l, $n = 32$, in plasma suspensions. There was no correlation between the intracellular Na^+ concentration and the ouabain inhibitable power.

DISCUSSION

Various glycolytic models have been proposed for energy metabolism in the erythrocyte (1, 13, 29, 30), in attempts to reconcile, predict and explain the events of cellular metabolism. The unifying theme of all the models is the regulation of glycolytic flux in the erythrocyte by the concentration of ATP and activity of the ATPases. The model of Rapoport et al. (29) and its extension (30) describe the time hierarchy of the glycolytic reactions and predict the assumption of a quasi-steady state for the flux of all metabolites except 2,3,-DPG about 0.5 to 2 hours after a perturbation of the erythrocyte system by addition of glucose after starvation. It also describes the increase in glycolytic flux with the activation of the Na^+, K^+ -ATPase also documented by others (28). If the steady state power values obtained for plasma and buffer suspensions of erythrocytes at about 0.5 and 2 hours in the present calorimetric determinations are related to the steady state flux of metabolites through the glycolytic pathway, then the decrease in power values in response to specific inhibition of Na^+, K^+ -ATPase is in accord with the predictions of the models of Rapoport et al. (30) and that of Ataullakhanov et al. (2). According to these models, inhibition of the Na^+, K^+ -ATPase activity should lead to an increase in ATP concentration. ATP is a known negative effector of the rate limiting enzyme phosphofructokinase (11), and its increase should decrease the flux of glucose and the rate of lactate production. The good correlation between power values and the rates of lactate production obtained by Minakami et al. (18) in their calorimetric studies of erythrocytes indicates steady state flux of metabolites generating

lactate and heat.

In the verification of a quantitative glycolytic model, Ataullakhanov et al. (2) found two types of regulation of energy metabolism in human erythrocytes. Specific inhibition of the $\text{Na}^+ \text{K}^+$ -ATPase by the action of ouabain led to increases in the concentration of glucose 6-phosphate (G6-P) and to decreases in the rates of glucose consumption and lactate production in most of their samples. In some samples they found, however, that the rate of glucose consumption did not change, although ouabain induced a decrease in the rate of lactate production and subsequently a decrease in the lactate/glucose ratio of 2. They argued that in the latter samples the decrease in the lactate glucose ratio from its value of 2 was only possible with increase in the flow of metabolites through the hexose monophosphate shunt.

Levin (15), using the LKB flow microcalorimeter, found no change in heat production rates when erythrocyte suspensions in buffer were treated with ouabain. Using the same type of flow microcalorimeter, we found, however, in an earlier study (11) an ouabain inhibitable component of heat production rates. In the present study and in our earlier study using the static calorimeter (24), we have consistently registered decreases in power values in all our plasma suspensions of erythrocytes treated with ouabain. This general decrease in power following inhibition of Na^+, K^+ -ATPase activity indicates a regulation of heat-producing processes in the erythrocyte by the Na^+, K^+ -ATPase. It also indicates that heat production by any probable

overflow of glucose through the hexose monophosphate shunt of the type proposed by Ataullakhanov et al. (2) cannot be large under our experimental conditions, cf calculations on page 75.

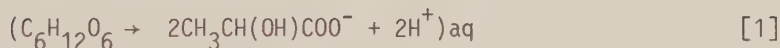
The ouabain inhibitable power of $(12 \pm 2) \mu\text{W/ml}$ and $(14 \pm 5) \mu\text{W/ml}$ for tris-HCl and plasma suspensions respectively obtained in this study are very similar. This suggests that the magnitudes of regulation of glycolysis by the Na^+ , K^+ -ATPase activity are similar during the two types of steady states 1 and 2 described for tris-HCl buffer and plasma suspensions of erythrocytes under Results. The steady state power time curve lasting for over three hours in tris-HCl buffer is worth noting. A steady state curve of such long duration is desirable where simultaneous determination of metabolite flux and power values are required, e.g. for evaluation of thermodynamic parameters and for probing enthalpy contributions from expected and unexpected processes.

The pattern of power-time curves obtained when erythrocyte glycolysis was inhibited with NaF, (Fig. 2) is in accordance with the findings of Minakami et al. (18) using flow microcalorimetry. In the present study, the linear relationship found between total power and fluoride inhibitable power indicate that the two parameters are quantitatively related. The mean value of $(38 \pm 5) \text{ mW/l}$ erythrocyte for the residual power representing $(46 \pm 4) \%$ of the total power obtained in this study agrees well with the value of 44% reported by Levin (15) and is close to the value of 50% obtained by Minakami et al. (18) for the residual rate of heat production after all lactate production has stopped. The samples from a patient with uremia and

one with liver disease gave high values of total power and fluoride inhibitable power. The values obtained using erythrocytes from normal subjects fall on the straight line relating the two parameters. This would indicate that the abnormal metabolism usually found in erythrocytes of liver disease (31) and uremia (17) are responses within the realm of normal metabolic regulation, perhaps similar to those in normal cells, inducible by e.g. an increase in pH or inorganic phosphate (12). More cases would need to be analysed for definite conclusions to be made.

THERMOCHEMISTRY OF ERYTHROCYTE METABOLISM

Expected sources of power, P , in erythrocyte suspensions. Let P_1 be the power expected from the glycolytic reaction



The corresponding enthalpy change, ΔH_1 , is -109.5 kJ/mol glucose (36).

Let P_2 be the power expected for the reaction



where H^+ is the liberated proton in reaction [1] and I^- is the proton acceptor. If the imidazole groups of hemoglobin are assumed to be the most probable proton acceptors within the erythrocyte (18), then ΔH_2 for reaction [2] is -27 kJ/mol (38).

Let P_3 be the power expected for the oxidation of glucose by the pentose phosphate pathway, PPP



The corresponding enthalpy change ΔH_3 is -2931 kJ/mol (37).

Let P_4 represent the sum of power contributions from undetermined processes.

$$\text{If } P_{\text{total}} = P_1 + P_2 + P_3 + P_4 \quad [4]$$

then $P_1 + P_2$ is the power associated with lactate production and $P_3 + P_4$ is the power in excess of that associated with lactate production.

As stated under results, the mean P value was (77 ± 3) mW/l cells, pH 7.35. The corresponding rate, V_g , for glucose consumption was $(0.270 \pm 0.008) \times 10^{-6} \text{ mol s}^{-1} \text{ l}^{-1}$. Assuming that 11% of the glucose flux is via the PPP (26), a glucose flux of $(0.030 \pm 0.001) \times 10^{-6} \text{ mol s}^{-1} \text{ l}^{-1}$ is estimated from the total flux V_g . Then $(0.240 \pm 0.0079) \times 10^{-6} \text{ mol s}^{-1} \text{ l}^{-1}$ is the direct flux through the glycolytic pathway. In addition, we can expect a contribution to the latter pathway from 5/6 of the glucose molecules passing through the PPP. Since the fate of this contribution is unknown quantitatively, it is excluded from the above glycolytic flux.

$$P_1 = (0.240 \times 109.5) \text{ mW/l} = 26 \text{ mW/l}$$

$$P_2 = (0.240 \times 2 \times 27) \text{ mW/l} = 13 \text{ mW/l}$$

$$P_3 = \left(\frac{0.03 \times 2931}{6} \right) \text{ mW/l} = 15 \text{ mW/l}, \text{ assuming that only the contribution from } {}^1\text{C of glucose is of significance to the observed power.}$$

Thus the power expected from lactate production, $P_1 + P_2$, = 39 mW/l. Using the experimental value of $P_{\text{total}} = 77 \text{ mW/l}$ and eq. [4], $P_3 + P_4 = 38 \text{ mW/l}$.

Comparison of the above calculated components of the experimental $P_1 + P_2$ values and the mean of the corresponding values, ΔP , obtained after enzyme inhibition shows that the values are similar. However, the component $P_4 = 23 \text{ mW/l}$ from calculation and representing 30% of the total power remains unaccounted for within the error limits of the quantities used for the calculations. The heat contribution from probable changes in the large pool of 2, 3-diphosphoglycerate (30) has not been estimated. This organic phosphate and formed protons have the role of modulating oxygen binding to hemoglobin (7).

It is concluded that specific enzyme inhibition can provide quantitative information on the origin of the total power observed in erythrocyte suspensions.

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TABLE 1. Data for erythrocytes suspended in plasma with and without NaF.

Sample	Hct	NO INHIBITOR		+ NaF		Δ pH	Δ P	P'x100
		pH	P	pH	P'			
			$\overline{mW/T}$		$\overline{mW/T}$		$\overline{mW/T}$	$\overline{P}$
1	42	7.36	73	7.38	34	0.02	39	47
2	48	7.43	82	7.49	43	0.06	39	52
3	41	7.42	104	7.53	45	0.11	59	43
4	49	7.40	92	7.45	40	0.05	52	43
5	50	7.31	64	-	29	-	35	45
6	51	7.33	87	7.51	37	0.18	50	43
7	41	7.42	79	7.45	35	0.03	44	44
8	46	7.39	72	7.41	37	0.02	35	51
9	41	7.45	83	7.46	41	0.01	42	49
*10	36	7.42	116	7.43	46	0.01	70	40
*11	32	7.45	148	7.45	56	0.00	92	38
Mean		7.39	82	7.46	38	0.06	44	46
$\pm$ SD		$\pm$ 0.05	$\pm$ 12	$\pm$ 0.05	$\pm$ 5	$\pm$ 0.06	$\pm$ 8	$\pm$ 4
n= 9								

* Samples 10 and 11 represent a case of liver disease and a case of uremia respectively. Their data are not included in the reported mean values.

FIGURE LEGENDS

Fig. 1. Power-time curve for plasma suspensions of erythrocytes.

Arrow denotes insertion of sample into the calorimeter.

Fig. 2. Power-time curve for plasma suspensions of erythrocytes in the presence of NaF.

Arrow denotes insertion of sample into the calorimeter.

Fig. 3. Total power as a function of sodium fluoride inhibitable power for erythrocytes suspended in plasma. The linear relationship is described by $y = 1.4X + 22$, $r^2 = 0.97$.

The symbols ●, ▲, □ represent results obtained for erythrocytes from healthy subjects, a patient with liver disease and a patient with renal disease, respectively.

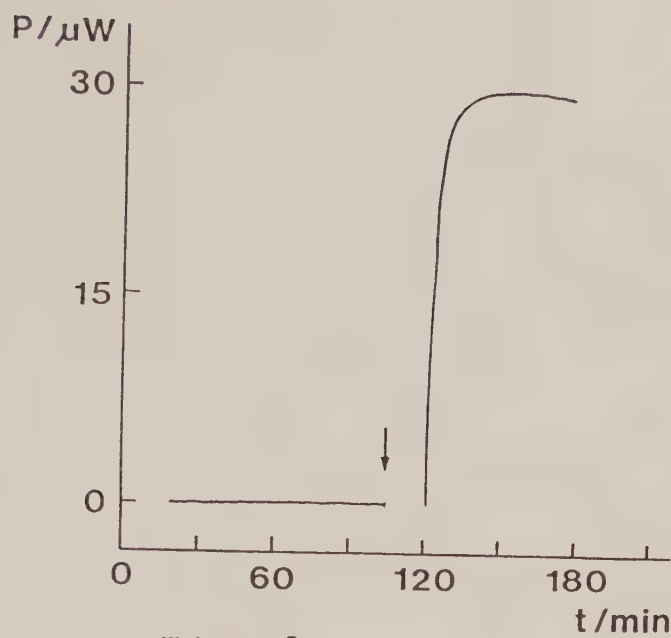


Fig. 1.

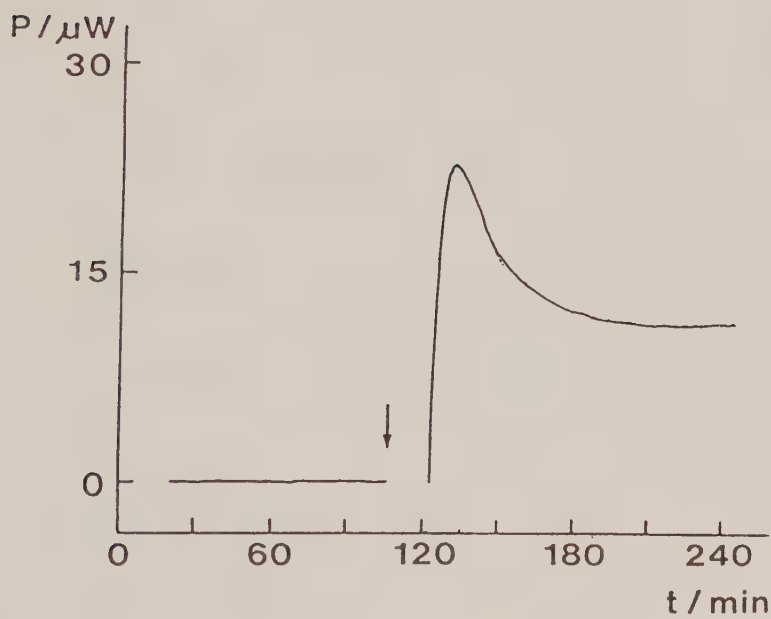


Fig. 2.

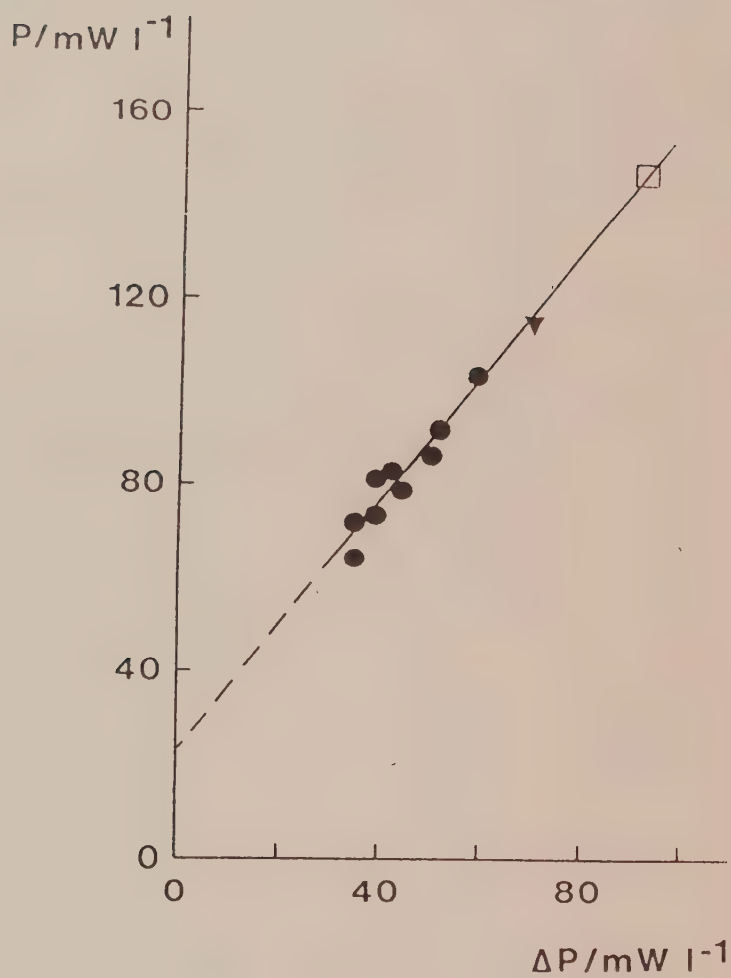


Fig. 3.

ERYTHROCYTE HEAT PRODUCTION IN HUMAN OBESITY. MICROCALORIMETRIC
INVESTIGATION OF SODIUM-POTASSIUM PUMP AND CELL METABOLISM.

Erythrocyte sodium potassium pump in obesity.

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ABSTRACT

Changes in over-all cellular metabolism, induced by specific inhibition of the Na,K-pump, were determined in erythrocytes from 33 normal and 25 obese subjects. Cellular metabolism was determined by measurement of heat production rates in erythrocytes suspended in plasma with and without the cardioactive glycoside, ouabain. Specific inhibition with ouabain induced the same decrease of the heat production rate in the two groups (14 ± 5 mW/l for normal subjects and 13 ± 5 mW/l for obese subjects). In neither group was there a correlation between the ouabain-inhibitable rate of metabolism and body weight. The present results do not give support to the suggestion that a defect in the Na,K-pump activity could exist in the erythrocyte of human obese subjects which could be of importance in the pathogenesis of the disease.

INTRODUCTION

Obesity is often due to excessive caloric intake. In many cases, however, the etiology of the disease is unclear. Several studies in the last few years have given support to the hypothesis that variations in metabolic efficiency could be of importance in the pathogenesis of the disease.^{1, 2}

Using animal models of obesity, several investigators^{3, 4, 5, 6} have found a deficient thyroid-dependent Na,K-ATPase activity, the enzymatic expression of the sodium potassium pump (Na,K-pump) in various tissues of obese (ob/ob) mice. Reduced activity of the Na,K-pump has been found in human obesity.^{7, 8, 9} However, there are also reports of normal¹⁰ and increased¹¹ Na,K-ATPase activity in erythrocytes and increased values in liver homogenates¹² from obese human subjects.

These controversial findings have been discussed in two recent studies^{10, 12} and correspondence,^{13, 14} where possible sources of errors in experimental procedures and data interpretation were pointed out. Nonetheless, the controversy remains unresolved, and the assessment and significance of the Na,K-pump in human obesity still require investigation.

Microcalorimetry has been proved suitable for the study of overall cellular metabolism of erythrocytes in health and disease.^{15, 16, 17, 18, 19, 20} On account of the high sensitivity of the technique,

small changes in metabolic rate can be quantified with good precision. In this study, by measurement of heat production rates, we have quantified the changes in over-all metabolism induced by specific inhibition of the Na,K-pump in erythrocytes of normal and obese subjects.

METHODS

Subjects

We have studied 25 obese patients who had no sign of endocrine disease. Their blood pressure was within normal limits, and none of them was on any drug. Their weights were stable. Sex, age and weight are given in Table 1. Thirty-three normal healthy adults with stable weight represented the control group. Sex, age and weight of the control group are given in Table 2. Subjects were classified as normal or obese by the use of Broca's index.

Preparation of Samples

Venous blood was collected into 10 ml Vacutainer tubes containing 143 USP units of sodium heparin. The isolation of erythrocytes from other blood cells was started within 10 minutes after the blood was drawn. The blood was centrifuged for 10 minutes at 650 g and the plasma and buffy coat were removed. Cell-free plasma was obtained by recentrifugation of the above plasma for 30 minutes at 3000 g. The erythrocytes were washed twice with about 3 parts of cold ($+4^{\circ}\text{C}$) 140 mM choline chloride solution. Each washing was followed by

centrifugation at 650 g for 10 minutes and removal of the choline chloride and buffy coat by gentle suction. One more wash was carried out in cell-free plasma, followed by centrifugation at 1000 g for 10 minutes and removal of the plasma and buffy coat. The erythrocytes were then resuspended in cell-free plasma to give a cell concentration of about 10%. In the final cell suspension, a mean value of 0.05×10^6 leucocytes /ml and 0.2×10^6 platelets/ml was noted. The contribution from these contaminating cells to the rate of heat production recorded for the erythrocytes was insignificant.

Just before calorimetric determinations of heat production rates were started, 25 μ l of a 15 mM solution of ouabain (Strophanthin G) from Sigma in 9 g/l NaCl was added to 1.0 ml of cell suspension. This ouabain concentration of 3.75×10^{-4} mol/l suspension, determined in a series of experiments, was the concentration of ouabain which gave maximum decrease in heat production rates and maximum increase in intracellular Na^+ as a result of inhibiting the pump.

Calorimetric Measurements

Two nearly identical twin microcalorimeters of the heat conduction type described elsewhere²¹ were used to register steady state heat production rates in the erythrocyte suspension. The cell suspension was enclosed in a 1.1 ml stainless steel ampoule. Calorimetric determinations were carried out under static conditions at 37 °C as described previously.¹⁸ The calorimetric registration of metabolic rates was carried out over two hours during which time

steady state rates of heat production were achieved. Heat production rates by the suspension of cells were expressed in mW per liter of packed cells (mW/l erythrocytes).

The methodological error, s , in heat production rates determined in an earlier study,¹⁵ simultaneously using two identical calorimeters and the same erythrocyte suspension, was 3.2 mW/l erythrocytes. The value for s was calculated from the expression

$$s = \sqrt{\frac{\sum \delta^2}{2n}}$$

where s is the difference between two simultaneous calorimetric determinations and n is the total number of pairs determined.

Other determinations

Hematocrit of the erythrocyte suspension was determined by the microhematocrit method. Leucocyte and platelet counts were made in a Bürker chamber. "t"-test was used for the statistical analysis of the results. Correlation lines were calculated by the method of least squares. Uncertainty limits are $\pm$ S.D.

Erythrocyte electrolyte

A direct method was used for the determination of the intracellular Na^+ , K^+ concentration of the erythrocytes. About 10 ml of venous blood was collected in Vacutain tubes containing 143 USP units of sodium heparin. The blood was immediately centrifuged at

600 g for 10 minutes and buffy coat plasma was removed. The erythrocytes were washed twice with 3 volumes of Choline Ringer buffer of the composition: choline chloride 146 mM; MgCl_2 1.0 mM; CaCl_2 , 1 mM; NaCl, 5 mM; orthophosphoric acid, 2.5 mM; pH 7.40. The cell suspension was centrifuged at 1800 g for 5 minutes and the Choline Ringer buffer discarded. The erythrocytes were then hemolysed by dilution with water followed by vigorous shaking. For sodium determinations, the erythrocytes were diluted 1:161 and for potassium, 1:14. Triplicate determinations of Na^+ , K^+ concentrations were carried out in Unicam emission flame photometers, Unicam Sp. 90. The intracellular Na^+ and K^+ concentrations so determined were corrected for residual buffer in extracellular fluid and for water loss during washing and centrifugation according to the method described.²²

At the end of the calorimetric determination, the cell suspension, with and without ouabain, was washed as described for fresh cells above, followed by flame photometric determinations. From these determinations, intracellular concentrations of Na^+ and K^+ were calculated for erythrocytes before calorimetry and erythrocytes with and without ouabain after calorimetry.

RESULTS

The results are given in Table 1 for the obese group and in Table 2 for the control group.

The mean values of heat production rates were 107 ± 13 (S.D.)

mW/l erythrocytes in the control group and 118 ± 17 mW/l erythrocytes in the group of the obese. The difference between the two groups is significant ($p < 0.05$). The heat production rates in erythrocytes suspended in plasma were greater than for those suspended in plasma containing ouabain. This was a general trend in all samples. The difference between the two rates in each sample was recorded as ouabain inhibitable rate of metabolism and consequently a measure of the rate of energy expenditure by the Na,K-pump. The ouabain inhibitable rate was 14 ± 5 mW/l for normal subjects and 13 ± 5 mW/l erythrocytes for obese patients. The difference between the two groups is not statistically significant ($p > 0.05$). In neither group did we find a correlation between change in the rate of energy expenditure due to the Na,K-pump and Broca's index ($p > 0.05$). Neither sex nor age was found to influence significantly the total rate of metabolism and the rate of energy expenditure by the pump in the two groups ($p > 0.05$).

Na and K determinations showed marked increases in intracellular Na^+ consequent to ouabain inhibition of the pump. Changes in intracellular K^+ were not as marked and were variable. Intracellular Na^+ and K^+ as well as changes in their concentrations after ouabain inhibition were the same in the normal and obese groups ($p > 0.05$).

DISCUSSION

In the last few years, the hypothesis has been advanced that a disturbance in cellular thermogenesis might lead to obesity.^{1, 2} The Na,K-pump has been suggested as the site of such a cellular defect.

Previous investigations of the Na,K-pump activity in obese animals and humans were based on measurements of Na,K-ATPase activity, the rate of active cation transport and of intracellular Na^+ concentration.^{3, 4,5, 6, 7, 8, 9, 10, 11, 12}

In studying the Na,K-pump as a possible pathogenetic factor in obesity, it is important to evaluate the extent to which any eventual derangement of function of the pump affects cellular thermogenic balance. In this regard, the only previous studies made in animals have been with indirect calorimetry by the measurement of rates of oxygen consumption.^{5, 6} No calorimetric evaluation of the Na,K-pump activity in obesity at the cellular level has been reported earlier.

Direct measurement of heat production rates has been used, however, in the last decade to investigate over-all metabolism in human blood cells.^{15, 16, 17, 18, 19, 20, 23}

Recently, microcalorimetry has been adapted to the measurement of heat production rates in isolated fat cells where significantly lower rates were found in obese than in lean subjects.²⁴ This finding was interpreted by the authors as giving support to the hypothesis that increased metabolic efficiency may play an important role in the pathogenesis of obesity. It is not known at present if the observed decrease in heat production rates is a consequence or a cause of obesity.

Although there are a number of studies establishing the relationship between the Na,K-pump activity and metabolic rate (as

determined by rates of glucose consumption, lactate and inorganic phosphate production), in erythrocytes in health,^{25, 26, 27, 28} and in spherocytic anemia,²⁹ there is no study of this relationship in human obesity. In erythrocytes from healthy subjects, a good correlation has been shown between the rates of heat production and lactate production (ATP synthesis).¹⁷

In the present work, we have studied in the erythrocytes of obese and normal subjects both the over-all rate of metabolism and energy expenditure due to the Na,K-pump activity. In view of the several known effectors of the Na,K-pump activity,^{30, 31} we have chosen experimental conditions close to the physiological by using fresh and intact cells suspended in autologous plasma. We put emphasis on the response of cellular metabolism to inhibition of the Na,K-ATPase activity and not on quantitative values of Na,K-ATPase activity per se, nor on the number of pump sites as was done in earlier studies.^{7, 8, 9, 10, 11} We have found no difference in the changes in metabolic rates induced by inhibiting the pump in erythrocytes from normal and obese subjects. However, basal heat production rates in erythrocytes from obese patients were significantly greater than those in erythrocytes from normal subjects. This finding is in qualitative agreement with the reports of elevated basal metabolic rates in obese subjects as estimated by indirect calorimetry,^{32, 33} and direct calorimetry.³⁴

We have obtained in this study a rather large spread in the ouabain inhibitable rates of heat production in erythrocytes from the two groups. This is consistent with the findings in earlier studies

on erythrocyte metabolism,^{25, 26, 28, 29} where variable effects of ouabain were found. In particular, our results are consistent with the findings in a recent work³⁵ where the inhibition of the Na,K-pump activity in human erythrocytes by ouabain induced two main types of energy metabolism. The ouabain effect was expressed as a decrease or unchanging rate of glucose consumption, while the rate of lactate production was decreased in the presence of ouabain for all the samples.

There is no agreement at present about the normal energy requirements of the Na,K-pump. In mammalian tissue, it has been estimated that about half the resting energy is used in transmembrane sodium transport.²⁷ Recently, microcalorimetric determinations of energy expenditure due to the active sodium-potassium transport have been made in the soleus muscle and brown adipose tissue of the rat and found to account for about 5-6% of the total energy flux.³⁶ For fresh erythrocytes, values of 15 and 25% of non-transport metabolism that are inhibitable by ouabain,^{25, 26} have been found to be associated with the sodium-potassium pump, percentages that are greatly altered by factors which change the flux of Na^+ and K^+ across the erythrocyte membrane.²⁵ In the present study we found that 11 and 13% of the total rate of heat production for obese and normal subjects respectively are associated with the Na,K-pump.

In conclusion, our present results speak against the hypothesis that a metabolic derangement secondary to a defective Na,K-pump activity in the erythrocyte is of importance in the pathogenesis of

human obesity.

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TABLE 1. Clinical and laboratory data on 25 obese patients.

Subject number	Age	Sex	Weight/ height	Broca's index	Heat production		Na,K-pump energy mW/l RBC
					mW/l RBC	- + ouabain	
1	53	F	94/162	1.5	102	86	14
2	30	F	110/169	1.6	117	106	11
3	50	M	115/181	1.4	119	114	5
4	60	F	92/164	1.4	121	111	10
5	49	M	107/179	1.4	112	103	9
6	34	F	98/180	1.2	110	101	9
7	55	F	102/167	1.5	118	112	6
8	41	F	87/168	1.3	110	98	12
9	64	F	90/172	1.3	106	90	16
10	46	M	131/177	1.7	113	100	13
11	67	M	140/160	2.3	130	119	11
12	47	M	112/173	1.5	99	89	10
13	62	M	104/174	1.4	108	100	8
14	43	F	120/165	1.8	156	137	19
15	48	F	100/164	1.6	119	105	14
16	34	M	103/181	1.3	165	146	19
17	39	F	102/161	1.7	107	93	14
18	56	F	103/170	1.5	100	86	14
19	18	F	135/169	2.0	102	93	9
20	39	F	89/158	1.5	139	125	14
21	65	F	168/160	1.3	108	97	11

22	52	M	99/161	1.6	98	86	12
23	44	M	140/176	1.8	132	114	18
24	36	M	163/179	2.1	121	94	27
25	46	F	136/157	2.4	126	111	15

Mean $\pm$ SD:

47 $\pm$ 12	114/169	1.6 $\pm$ 0.3	108 $\pm$ 15
15F/10M		118 $\pm$ 17	13 $\pm$ 5

TABLE 2. Clinical and laboratory data on 33 normal subjects.

Subject number	Age	Sex	Weight/ height	Broca's index	Heat production		Na,K-pump energy
					mW/l RBC	↓ ouabain	mW/l RBC
1	32	F	57/168	0.8	130	112	18
2	39	M	76/183	0.9	137	114	23
3	30	F	55/169	0.8	126	110	16
4	39	M	80/185	0.9	118	105	13
5	55	F	56/163	0.9	99	85	14
6	36	M	82/186	1.0	97	82	15
7	25	M	70/180	0.9	98	88	10
8	50	F	65/166	1.0	108	98	10
9	28	F	57/178	0.7	112	87	25
10	30	M	69/178	0.9	127	109	18
11	30	F	61/170	0.9	124	107	17
12	27	M	67/172	0.9	98	86	12
13	29	M	72/180	0.9	106	86	20
14	28	F	56/159	1.0	102	90	12
15	42	M	83/183	1.0	101	90	11
16	27	F	51/156	0.9	108	86	22
17	24	F	55/175	0.9	90	80	10
18	29	F	57/162	0.9	108	90	18
19	56	F	65/168	1.0	74	66	8
20	18	F	59/168	0.9	92	88	4
21	44	M	90/192	1.0	110	100	10

Heat Production in Human Blood Lymphocytes. A Methodological Study.

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Running headline: Heat Production in Human Blood Lymphocytes

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ABSTRACT

Heat production rates (thermal power) in peripheral blood lymphocytes from healthy subjects were determined under some defined experimental conditions in an attempt to establish by microcalorimetry a basal metabolic reference range for lymphocytes in the non-activated state. The effects of cell isolation method, the presence of other types of blood cells, cell concentration, temperature, pH and type of suspension medium on the rate of heat production by lymphocytes were evaluated. The results indicate that microcalorimetry is suitable for monitoring the metabolism of these cells with good precision in the physiological range of cell concentration.

Key-words: Heat production rate, metabolism, microcalorimetry, power, power-time curve.

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INTRODUCTION

Accurate and precise baseline data on the metabolism of the mature, non-proliferating lymphocyte are few because of its low rate of metabolism and the difficulty of obtaining pure lymphocyte preparations. Earlier attempts by Górski et al (7) and Krakauer et al (12) to apply flow microcalorimetry to the study of lymphocyte metabolism and activity in the immune system were limited by a number of methodological problems. The intimate interactions between the various cell types in blood and the heterogeneity of leukocyte populations made the complete isolation and metabolic characterisation of lymphocytes somewhat less than successful as reflected in the large variability of heat production rates reported earlier for human lymphocytes in health (1, 7). Heat production rates in lymphocyte suspensions in plasma or buffer at physiological concentrations are low, and in these earlier studies calorimetric measurements were made close to the detection limit of the recording system. Static microcalorimeters of the heat conduction type (24), used earlier to monitor blood cell metabolism by Monti et al (15) and in the present work, have a sensitivity which is higher than that of the earlier flow version.

This study was carried out to determine by microcalorimetry the optimum conditions for recording metabolic rates in peripheral blood lymphocytes *in vitro*, to explore the time course and significance of typical calorimetric signals (power-time curves) and to search for systematic errors inherent in the measurements. Such baseline data are required for using microcalorimetry to

- i determine the thermochemical properties of lymphocytes at the molecular level;
- ii study lymphocyte interaction with other blood cells;
- iii evaluate lymphocyte activity in the immune system;
- iv characterise metabolic properties of lymphocytes in proliferative diseases of the lymphoid system, knowledge of which could be useful in the classification of malignant lymphomas (2, 18).

MATERIALS AND METHODS

About 40 to 60 ml of venous blood were obtained from healthy non-fasting volunteers between the hours of 0800 and 1200. One set of blood samples was collected in heparin as anticoagulant, 14.3 USP units per ml blood, while the other was defibrinated by gently shaking with glass beads. Lymphocytes were isolated from each set by density gradient centrifugation in Ficoll-Paque medium (Pharmacia, Uppsala) by the method of Böyum (3). The cells at this stage of preparation contained varying amounts of phagocytic cells, mainly monocytes and granulocytes. The phagocytic cells were removed from the samples by two different methods. In method A, Fe method (13), the "impure" lymphocytes were suspended in Hanks balanced salt solution (Flow Laboratories) containing 20% autologous plasma mixed with iron powder (Merck) and incubated at 37 °C under gentle rotation. After 40 minutes, the phagocytic cells, which had ingested the iron particles, were removed with a magnet. The second method B, Sephadex G-10 method, was that described by Jerrels et al (11). Lymphocytes isolated by

the method of Böyum were suspended in Hanks balanced salt solution containing 20% plasma and applied to a Sephadex column at 37 °C. The phagocytic cells adhered to the column while the lymphocytes were eluted from the column with the suspension medium. The cells at the end of procedures A and B were washed twice with 50 ml Hanks balanced salt solution. This washing procedure and the large volume used were necessary for removal of thrombocytes and products of phagocytosis in solution. The cells were then resuspended in cell-free plasma or buffer at concentrations between 0.5×10^6 and 6.0×10^6 cells/ml just before calorimetry. Cell-free plasma was obtained by recentrifugation of plasma at $8000 \times g$ for 15 minutes and stored at 25 °C in gas-tight tubes. The buffer consisted of RPMI 1640 medium (19) containing 20 mM Hepes (Flow Laboratories) and 36 mM NaHCO_3 , pH 7.50-7.60.

Lymphocytes isolated as above contained less than 0.02×10^6 erythrocytes per ml, less than 0.3×10^6 platelets per ml and less than 0.05×10^6 phagocytic cells per ml at the highest lymphocyte concentration.

Assuming the normal thermal power for these cells as 59 ± 8 fW per platelet, 3.5 ± 1.0 pW per phagocytic cell (granulocytes or monocytes), and 9.0 ± 0.8 fW per erythrocyte (1, 17), none of these cell types was expected to contribute significantly to the thermal power observed for lymphocytes at all the cell concentrations studied. No further attempts were therefore made to remove these cell quantities from the preparations.

Absolute cell counts were carried out in a Bürker chamber on

duplicate dilutions of lymphocytes in 0.1 % Trypan blue in 0.9% NaCl just before calorimetric measurements. Only viable cells were enumerated for calculation of power values. Viability of the lymphocytes was determined by the Trypan blue exclusion test before and after calorimetry.

Calorimetric measurements

Two identical microcalorimeters of the thermopile heat conduction type (24) were used. 1.0 ml plasma or lymphocyte suspension in plasma or buffer was enclosed in a 1.1 ml stainless steel ampoule and heat production rates (power values) were recorded under static conditions as described in a previous report (16). Steady state heat production rates (indicated by power-time curves of slope zero) were achieved 30 minutes after the ampoules were inserted in the calorimeter and power-time curves were monitored for at least another 30 minutes. In some experiments the power-time curves were recorded for as long as the steady state lasted, in order to obtain information on the maximum duration of the steady state for the particular experimental conditions defined by pH, cell concentration, type of suspension medium and heterogeneity of the cell suspension. In the results, only steady state heat production rates lasting up to 1 hour after sample introduction into the calorimeter are reported, except in determinations using lymphocyte suspensions containing phagocytic cells. For such suspensions, non-steady state power values obtained one hour after samples were introduced into the calorimeter are reported. At the end of the calorimetric determination, the pH of the sample aspirated from the bottom of the ampoule was registered using a Radiometer PHM 52 digital pH meter with type G297/G2 micro capillary

electrodes.

Design of the experiments

Suspension of the lymphocytes. Autologous plasma and buffer containing 36 mM NaHCO_3 were used for lymphocyte suspensions. At the end of the preparation, the lymphocytes were stored at concentrations of $(50 \text{ to } 10) \times 10^6/\text{ml}$ in 0.9 g/l NaCl at 25 °C. They were suspended in plasma or buffer just before calorimetric determinations. After calorimetry, the total power registered for each plasma suspension of lymphocytes was corrected for its plasma contribution. The heat production rate per lymphocyte, P, was then calculated from the corrected power value and the corresponding cell count.

Variation of pH. The dependence of power values on the pH of the plasma suspension medium was investigated in the range 7.00 to 8.20. Plasma pH was adjusted to values in this range by adding (0 to 100) μl of 0.1 M HCl or NaOH in 0.9 g/l to 1.5 ml portions of plasma. These adjustments were made 5 to 10 minutes before calorimetric measurements.

Variation of temperature. For temperature effects, pair-wise determinations of power values were carried out simultaneously in two calorimeters at temperatures of [(42.00, 37.00); (32.00, 37.00); (25.00,, 37.00)] ± 0.03 °C. For these determinations, plasma was used as suspension medium without addition of HCl or NaOH.

RESULTS

Power-time curves

The calorimetric signal (power-time curve) was recorded over a period of not less than one hour. Both steady-state and rapidly decreasing power-time curves of the types indicated in Figures 1a and 1b respectively were observed for pure lymphocytes suspended in plasma or buffer. Steady state power-time curves of the type shown in Figure 1a, with a duration of at least 30 minutes, were always observed for pure lymphocyte suspensions in plasma or buffer at concentrations less than 5.0×10^6 cells per ml. Decreasing power-time curves of the type shown in Figure 1b were often obtained for pure lymphocyte suspensions, when concentrations were greater than 6.0×10^6 cells/ml. When the lymphocyte preparation contained more than a few per cent phagocytic cells, decreasing power-time curves were often obtained even at concentrations below 3.0×10^6 per ml. Such decreasing curves have been observed generally for granulocytes suspended in plasma or buffer with and without opsonised particles at all concentrations down to 0.5×10^6 cells/ml (unpublished).

Sensitivity and reproducibility of determinations

Calorimetric. The sensitivity of the calorimetric recording system was $0.3 \mu\text{W}$ with a baseline stability of the same magnitude over 24 hours. Table 1 shows P values obtained from two simultaneous determinations of the same lymphocyte preparation suspended in plasma, using identical calorimeters. The estimated methodological error, s , was 2% as calculated from the relationship

$$s = \sqrt{\frac{\sum d^2}{2n}} \quad (1)$$

where d is the difference between pairs of determined P values and n the number of samples. Uncertainties in power values and related parameters are $\pm$ SD.

Cell count. Within the concentration range of $(2.0 \text{ to } 6.0) \times 10^6$ cells/ml, a methodological error of 8% was calculated for 15 duplicate lymphocyte counts. The corresponding error in the range $(0.5 \text{ to } 2.0) \times 10^6$ cells/ml was 20%.

Effect of storage

When lymphocytes suspended in plasma were stored unstirred in a test tube for 4 hours at 25°C followed by calorimetry at 37°C , the P value decreased $(5.0 \pm 0.8)\%$ per hour. If, however, the cells were stored in a balanced salt solution or 0.9 g/l NaCl without glucose and resuspended in plasma or buffer just before calorimetric measurements, the P values and viability remained unchanged during 4 hours. These data were obtained from a set of experiments with lymphocytes from 5 different donors. Subsequently all calorimetric determinations were carried out within 4 hours after preparation of the lymphocytes which were stored in NaCl solution as described above.

Effect of the degree of purity of lymphocyte preparations

The standard method of Böyum (3) in the present study gave lymphocyte preparations with varying quantities of phagocytic cells, some (2 to 37)% of the total number of enumerated cells. Table 2 shows the dependence of power per lymphocyte on the amount of phagocytic cells present in such preparations. Since decreasing power-time curves were a regular feature of the preparations, especially at high

cell concentrations, the P values in Table 2 obtained using cells in the concentration range $(3.9 \text{ to } 5.9) \times 10^6$ cells/ml were non-steady-state values. The results indicate an increase in power per cell with increase in the number of phagocytic cells. Both the Fe method and the Sephadex G-10 method were effective in obtaining lymphocyte preparations completely free from phagocytic cells as verified by microscopy of esterase-stained thin smears of the preparations (22). The prepared cells showed the morphology of the small lymphocyte by microscopy of Giemsa-stained smears. The two isolation methods also gave similar lymphocyte viability of $(98 \pm 2)\%$ by the Trypan blue exclusion test. The Fe method was chosen in all but one set of experiments because of its comparative simplicity. As shown in Table 3, the same P value (1.8 ± 0.3) pW/cell was obtained for lymphocytes isolated by the Sephadex G-10 and Fe methods.

Effect of anticoagulation

The P values of lymphocytes in plasma prepared from heparinised and defibrinated blood were (1.8 ± 0.3) pW/cell and (2.7 ± 0.5) pW/cell, respectively, as shown in Table 4. The difference is significant, $P < 0.005$. Heparinised blood was found to be more convenient to handle and gave higher lymphocyte yields. In contrast to lymphocytes from defibrinated blood, lymphocytes from heparinised blood also often gave steady-state power-time curves at higher cell concentration. Therefore heparinised blood was used in most of the experiments.

Effect of pH of the lymphocyte suspension

Fig. 2 shows a typical pH profile of power values in the pH range

7.00 to 8.20 for plasma suspensions of purified lymphocytes from one donor. When plasma was used as suspension medium without addition of acid or alkali, the pH of the suspension was 7.85 ± 0.14 . For each of pure lymphocyte preparations from 5 donors, power values were determined at 2 or 3 different pH in the range 7.20 to 7.50. At each of the pH, the power P_{exp} was expressed as a percentage of the power P^0 at pH 7.40, as was done in our previous calorimetric studies of platelets (17) and erythrocytes (16). Fig. 3 shows a plot of $(\frac{P_{\text{exp}}}{P^0} \times 100)$ as a function of pH. The straight line shown was evaluated by the method of least squares, $r^2 = 0.95$. From the straight line, the expression

$$P_{\text{exp}} = P^0(1 + 0.78 \Delta\text{pH}) \quad (2)$$

was deduced, where

P_{exp} is the power per lymphocyte at experimental pH

P^0 is the power per lymphocyte at pH 7.40

ΔpH is the increase in pH above 7.40.

From these determinations using samples from 5 blood donors, P^0 values were obtained. The P values of 5 other lymphocyte preparations at one pH value close to 7.40 were determined and their P^0 values calculated from equation 2. A mean value of (1.6 ± 0.3) pW/cell for the 10 samples represented the P^0 for plasma suspension of the lymphocytes.

Effect of cell concentration

As can be seen in table 5, P values for plasma suspensions tend

to increase at the lower cell concentration when pair-wise determinations at two lymphocyte concentrations are compared. However, if the mean of all P values of lymphocytes from different donors in this study are compared in the range $(2.0 \text{ to } 6.0) \times 10^6$, the spread is less than 10% of the mean. This is the case for lymphocytes obtained from both heparinised and defibrinated blood.

Effect of suspension medium

The mean power value for cell free plasma was $(1.7 \pm 0.9) \mu\text{W/ml}$, range $(0.0 - 2.9) \mu\text{W/ml}$, $n = 19$. For cell-free RPMI buffer medium, zero power value was obtained in each determination. Table 6 shows P^0 values for lymphocytes from different preparations suspended in plasma and in the buffer medium, RPMI. The mean P^0 value for lymphocytes suspended in plasma was $1.7 \pm 0.2 \text{ pW/cell}$, $n = 4$. For lymphocytes suspended in buffer, mean P^0 was $1.6 \pm 0.2 \text{ pW/cell}$, $n = 6$.

Effect of temperature

Figure 4a shows the dependence of heat production rate on temperature for pure lymphocytes suspended in plasma. At each of the temperatures, P values were determined using lymphocytes from two different donors. The power, P, at temperatures 25°C , 32°C and 42°C was expressed as percentages of the power P^0 at 37°C . Figure 4b represents an Arrhenius plot of $\log \frac{P}{P^0} \times 100$ (log relative rates) as a function of $\frac{1}{T} \text{ K}^{-1}$. There is a linear relationship between the two parameters in the temperature range 25°C - 37°C . From the slope of the line, an apparent activation energy of 62.9 kJ/mol was calculated.

DISCUSSION

In a study of immune properties of lymphocytes, Górski et al (7), using an LKB flow microcalorimeter, obtained heat production rates of (3 to 10) pW/cell for mature lymphocytes from healthy subjects. Bandmann et al (1), using the present type of ampoule calorimeter of higher sensitivity and improved quantitative sampling, obtained rates of (2.2 ± 1.4) pW/cell for a plasma suspension of human lymphocytes prepared from defibrinated blood. This value is in agreement with our value of (2.7 ± 0.5) pW/cell for plasma suspension of lymphocytes from defibrinated blood. We do not understand the significance of the lower P value, (1.8 ± 0.3) pW/cell, for heparinised blood lymphocyte in this study. In a calorimetric study of platelet metabolism by Monti et al (17), significantly lower P values were found for platelet suspensions when heparin replaced citrate as anticoagulant.

A few important features distinguish our study from the earlier works and partly account for the comparatively low standard deviation of the mean for P values obtained in lymphocytes from different donors.

The method of Böyum, which was used by Bandmann et al (1) gave in the present study a lymphocyte preparation with variable quantities of phagocytic cells. Phagocytic cells are known to respond to various stimuli (organic surfaces, cellular materials, dead cells, opsonised particles, etc.) with increase in glycolysis and oxidative metabolism. Such responses have been demonstrated by microcalorimetry as marked and variable increases in heat production rates (4, 6, 7, 25) which if not accounted for could introduce large systematic errors into

the power observed for lymphocytes. The high P values shown in Table 2 for lymphocyte preparations containing phagocytic cells illustrate this point. By using the phagocytic properties of the cells, in additional steps of cell preparation, lymphocytes essentially free from phagocytes were obtained in our study.

Krakauer et al (12) reported heat production rate of 8 pW/cell for fresh equine lymphocytes, a value that rapidly decreased and reached a steady state value of 1.0 pW/cell in 24 hours. They could investigate the immune response of the lymphocytes to different mitogenic agents only after 24 hours. This is in contrast to the rapid achievement of steady state and the low variability of heat production rates obtained in this study which make early metabolic characterisation of freshly prepared lymphocytes possible if required. We have earlier determined the power-temperature relationships for erythrocytes (16) and platelets (17). In both cases linear Arrhenius plots were found over the temperature range 25 °C - 41 °C, leading to apparent activation energy values of 83 kJ/mol and 52 kJ/mol, respectively. For lymphocytes we find an intermediate value, 63 kJ/mol, for the range 25 °C - 37 °C. However, there is a significant break in the linear relationship between 37 °C and 42 °C, suggesting that the nature of the rate-limiting step changes in this temperature interval.

Several workers (5, 9, 20, 21) have obtained results which suggest that lymphocytes show a significant density dependent inhibition of O₂ consumption ("crowding effect"), whereas others (8, 23) have reached a different conclusion. These metabolic studies were

carried out over a rather wide range of cell concentrations (0.1 to 110×10^6 /ml. The calorimeter technique we have used in this work is not ideal for studies of "crowding effects". In a static ampoule the cell quantity is well-defined but, due to sedimentation, the cell concentration is different in different parts of the ampoule and varies with time. However, the fact that we observe good steady state values during the time period when the sedimentation is expected to occur does suggest that the "crowding effect" is small or absent. We reach the same conclusion from the observation that we do not find any significant trend in P values with variations in cell concentrations in the range $(2-6) \times 10^6$ cells/ml. However, when we compared results of pair-wise experiments with cells of concentrations between about 0.5×10^6 cells/ml and 4×10^6 cells/ml (obtained from the same preparation), the P values were slightly higher for the lower concentrations. The accuracy of P^0 values obtained for concentrations as low as 0.5×10^6 cells/ml can, however, easily be impaired by systematic errors, e.g. connected with the comparatively large power value for plasma and low precision of cell count. A calorimetric investigation of a possible "crowding effect" in lymphocyte suspensions should preferably be performed with cells in a stirred reaction vessel with special care taken to avoid systematic errors connected with as yet undefined processes in the suspension medium. Such experiments are in progress at this laboratory.

Lymphocytes isolated by the procedures in this study are known to consist of subsets that have different surface properties and are functionally heterogeneous (10). No attempts have been made to classify and identify the different subsets in our preparations.

However, within the range of cell concentration and experimental conditions used in this study, useful clinical information could be obtained from determined rates of heat production in chronic lymphocytic leukemia (2) and non-Hodgkins lymphoma (18).

This methodological work is part of a long-term systematic study of lymphocytes and other white blood cells by microcalorimetry. The aim has been to lay out experimental procedures and conditions for characterising various aspects of lymphocyte metabolism on a molecular level and to study their interaction with other blood cells for clinical application. The small interdonor variation in thermal power per lymphocyte obtained in this study indicates that the mature lymphocyte of peripheral blood is a cell with defined basal metabolism amenable to precise calorimetric studies.

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TABLE 1.

Double determinations of P values for defibrinated blood lymphocytes in plasma. The lymphocytes were purified by the Fe method.

Sample	Calorimeter Designation	Number of cells 10^6	pH	P $\overline{pW/cell}$
1	A	5.75	7.71	2.71
	B		7.72	2.58
2	A	6.55	7.75	2.38
	B		7.73	2.35
3	A	4.35	7.83	2.84
	B		7.83	2.95
4	A	6.15	7.94	2.41
	B		7.94	2.33
5	A	3.00	7.72	1.61
	B		7.76	1.65
6	A	5.23	7.94	2.21
	B		7.92	2.08
7	A	4.60	7.90	1.99
	B		7.88	1.95
8	A	4.65	7.71	2.07
	B		7.71	1.72

9	A	5.05	7.87	2.10
	B		7.87	2.06
10	A	4.45	8.04	2.96
	B		8.04	2.97

TABLE 2

Effect of degree of purity of the lymphocytes.

Sample	Number of cells	pH	Lymphocytes*	P**
	10^6		%	pW/cell
1	5.18	7.72	100	2.1
2	4.03	7.75	100	2.7
3	4.68	8.03	100	2.7
4	5.88	7.72	100	2.0
5	3.90	7.90	98	3.4
6	5.20	7.83	98	3.0
7	4.90	7.77	96	2.3
8	5.10	7.89	89	2.6
9	4.30	7.82	84	3.5
10	5.20	7.68	75	3.6
11	4.65	7.70	71	3.7
12	4.06	7.72	63	3.9

* Percentage of lymphocytes in the leukocyte suspension (monocytes, granulocytes and lymphocytes).

** P refers to the power per leucocyte in the plasma suspension. The samples were obtained from defibrinated blood of 12 different donors by the method of Böyum. P values were determined using samples 5-12 without further purification, while samples 1-4 were purified by the Fe method.

The P values of samples 5-12 were non-steady-state values obtained exactly 1 hour after samples were introduced into the calorimeter.

TABLE 3

Effect of method of purification on heat production rate per cell, P, in lymphocytes suspended in plasma.*

Method	n	Number of cells		pH	P
		10^6			
		Range	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Sephadex G-10	4	2.0-5.6	3.3 $\pm$ 1.6	7.95 $\pm$ 0.10	1.8 $\pm$ 0.2
Fe	8	3.2-5.4	4.5 $\pm$ 0.8	7.80 $\pm$ 0.14	1.8 $\pm$ 0.3

* Lymphocytes were obtained from heparinised blood of different donors and purified by the Fe method.

TABLE 4

Effect of method of anticoagulation on P values of lymphocytes in plasma.*

Type of blood	n	Number of cells		pH	P
		10 ⁶			
		Range	Mean $\pm$ SD	Mean $\pm$ SD	Mean $\pm$ SD
Heparinised**	8	3.2-5.4	4.5 $\pm$ 0.8	7.80 $\pm$ 0.14	1.8 $\pm$ 0.3
Defibrinated	6	2.6-4.4	3.6 $\pm$ 0.7	7.84 $\pm$ 0.07	2.7 $\pm$ 0.5

* Lymphocytes were purified by the Fe method.

** Same as data given in table 3 under Fe method.

TABLE 5

P values for lymphocytes suspended in plasma at high and low cell concentrations.*

Sample	Number of cells	pH	P
	10^6 pW/cell		
1	0.90	7.93	2.6
	4.40		2.2
2	0.80	7.84	2.4
	4.35		1.9
3	0.70	7.73	3.4
	3.35		2.8
4	0.40	7.78	3.5
	2.60		2.7
5	0.69	7.84	3.7
	3.90		3.3
6	0.50	7.89	4.7
	3.10		3.2

* Lymphocytes were obtained from defibrinated blood of 6 different donors and purified by the Fe method. Data for the higher concentrations are the same as those given in Table 4 for defibrinated blood.

TABLE 6

P^0 values for lymphocyte suspensions in plasma and buffer.*

Samples	Lymphocytes suspended in buffer		Lymphocytes suspended in plasma	
	Number of cells 10^6	P^0 $\frac{pw}{cell}$	Number of cells 10^6	P^0 $\frac{pw}{cell}$
1	2.65	1.9	1.70	1.9
2	3.51	1.4	3.51	1.5
3	5.00	1.6	5.75	1.6
4	-	-	2.40	1.8
5	1.40	1.6	-	-
6	2.80	1.4	-	-
7	2.50	1.5	-	-
Mean $\pm$ SD		1.6 $\pm$ 0.2	Mean $\pm$ SD	1.7 $\pm$ 0.2
n = 6			n = 4	

* Lymphocytes were prepared from heparinised blood by the Fe method.

The buffer medium was RPMI 1640 (Flow Laboratories) containing hepes, 20 mM and NaHCO_3 , 36 mM. P^0 refers to power value at pH 7.40.

FIGURE LEGENDS

- Fig. 1a Steady state power-time curve usually obtained for lymphocyte suspensions in the concentration range $(0.5 \text{ to } 5.0) \times 10^6$ cells/ml. ↓ Sample ampoule inserted into the calorimeter.
- Fig. 1b Decreasing power-time curve usually obtained for lymphocytes containing phagocytic cells and for pure lymphocytes at concentrations greater than 6.0×10^6 cells/ml. ↓ Sample ampoule inserted into the calorimeter.
- Fig. 2 A typical pH profile of P values in the pH range 7.00 to 8.20 for plasma suspensions of lymphocytes.
- Fig. 3 The dependence of P values on pH of lymphocyte suspensions in plasma. P values are expressed as per cent of corresponding value P^0 at pH 7.40.
- Fig. 4a The dependence of P values of lymphocyte suspensions in plasma on the measurement temperature. P values are expressed as per cent of the corresponding value P^0 at 37°C .
- Fig. 4b Arrhenius plot of $\log \frac{P}{P^0} \times 100$ as a function of reciprocal temperature in the range 298 K to 315 K. From the slope of the straight line portion an apparent activation energy of 62.9 kJ/mol was obtained.

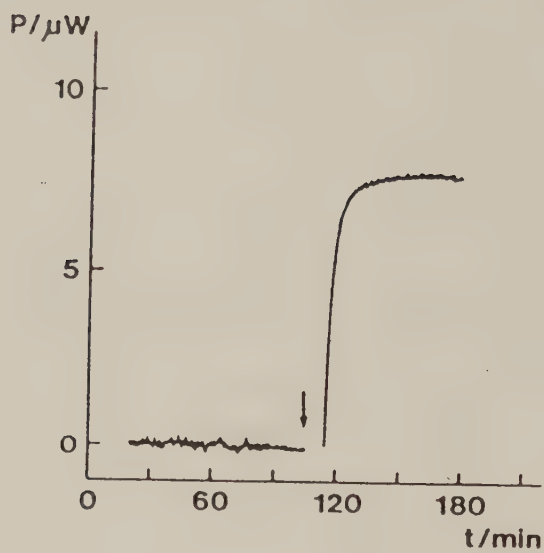


Fig. 1a

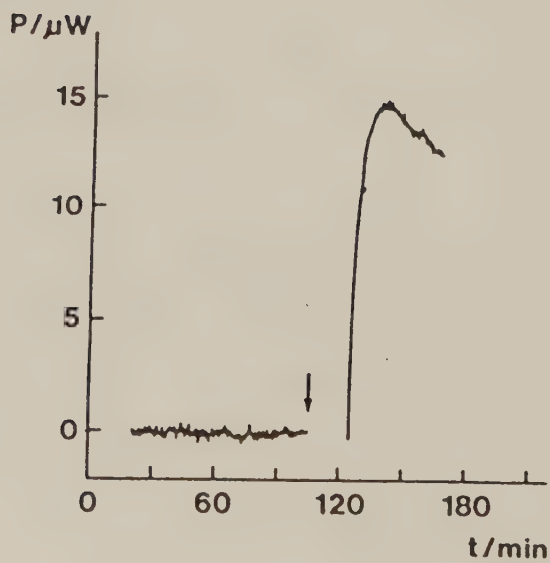


Fig. 1b

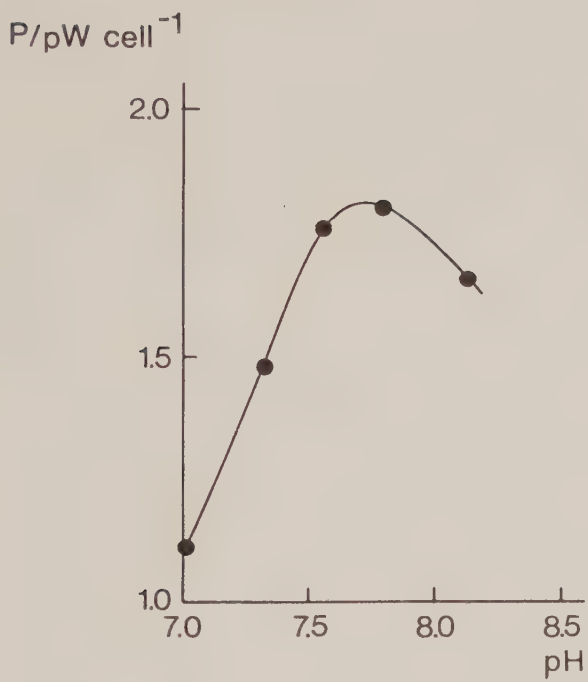


Fig. 2

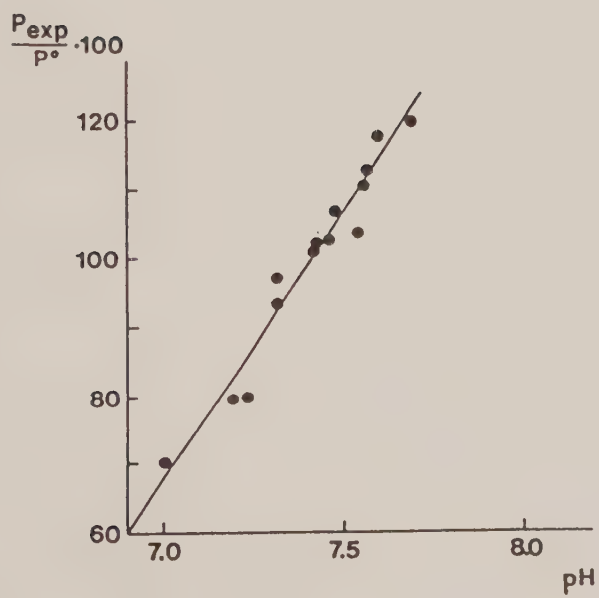
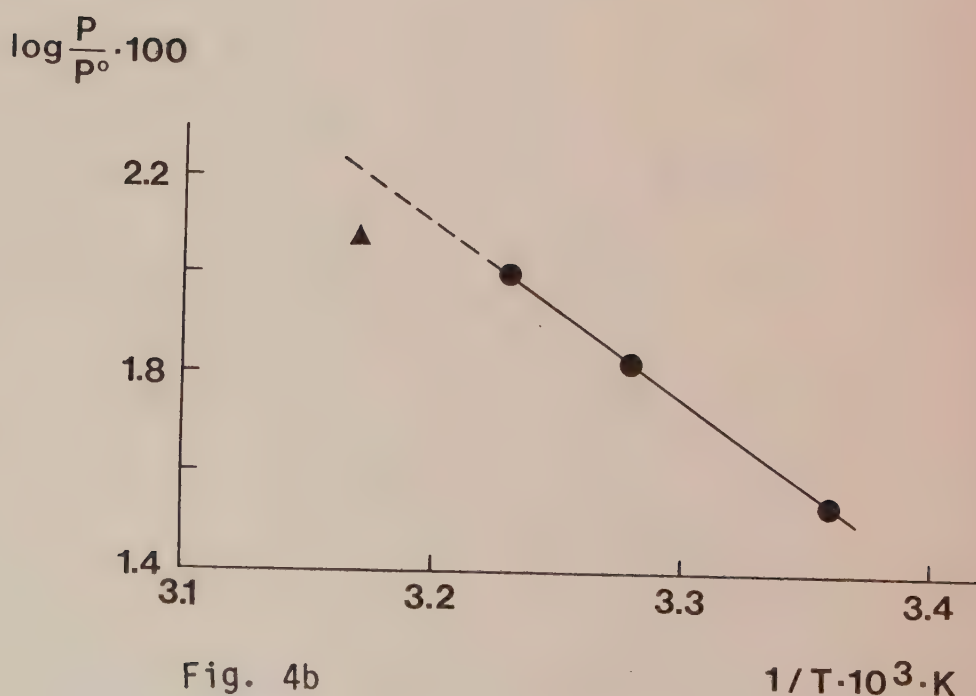
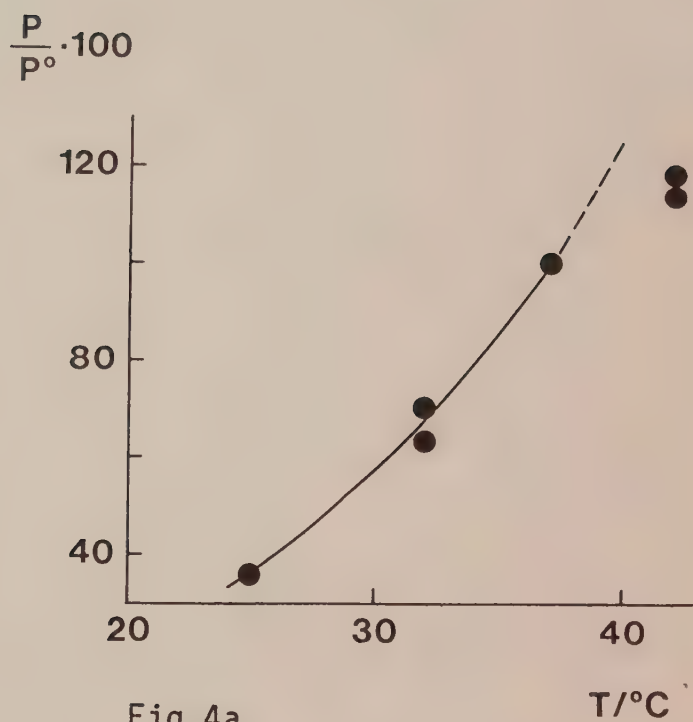


Fig. 3



Heat Production by Lymphocytes in Chronic Lymphocytic Leukaemia

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Using microcalorimeters of the thermopile conduction type heat production was measured in lymphocytes from peripheral blood in 8 normals and 10 patients with chronic lymphocytic leukaemia (CLL). The heat production per CLL lymphocyte was lower (1.8 pW/cell) than that found in normal lymphocytes (2.6 pW/cell). Due to the high numbers of lymphocytes in the peripheral blood the estimated heat production of the intravascular lymphocyte pool in CLL was considerably higher than in normals. Since the circulating lymphocytes constitute a minute fraction of the total lymphoid mass in CLL it is suggested that the accumulation of metabolically active lymphocytes in blood and tissues may explain the common clinical signs of hypermetabolism in this disease. The results also indicate that calorimetry may be a useful technique for metabolic studies in suspensions of malignant cells.

Key words: CLL – heat production – hypermetabolism – lymphocytes

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Sweating, loss of weight and an increased basal metabolic rate unassociated with hyperthyroidism are often found in CLL. However, the mechanisms bringing about the hypermetabolic state are incompletely known (Hansen 1973).

Calorimetry has lately been applied to the investigation of metabolic processes in blood cells and has proven to be a suitable technique for measuring the total metabolic activity of a cell population (Monti & Wadsö 1978). In the present study the heat pro-

duction in lymphocytes has been measured in normals and in patients with CLL. An attempt has been made to evaluate the metabolic activity of the lymphocyte mass in CLL.

MATERIALS AND METHODS

Patients and controls

10 patients with elevated WBC counts, $13\text{--}210 \times 10^9/l$, due to typical CLL were selected without regard to Hb concentration, platelet counts, degree of lymph node enlargement or spleen size.

Untreated patients as well as patients under treatment with alkylating agents were included. 8 healthy volunteers served as controls.

Preparation of samples

Lymphocytes were isolated from defibrinated whole blood by density gradient centrifugation in Ficoll-Paque medium (Pharmacia, Uppsala) by the method of Böyum (1968). Contaminant phagocytizing cells were removed by incubating the lymphocyte suspension in plasma with iron filings at 37° C under rotation ½ h and removing the iron and adherent phagocytic cells with a magnet. The lymphocytes were washed twice in phosphate buffer, pH 7.40 and resuspended in autologous plasma which had been recentrifuged at 8,000 × g. The lymphocyte concentration was adjusted to about 4 × 10⁹ cells/l and counting of lymphocytes in each final cell suspensions was performed in a Bürker chamber just before calorimetric measurements. The pH of the final lymphocyte suspension taken directly from the calorimeter ampoule at the end of 1 h incubation at 37° C was determined using a Radiometer capillary electrode pH meter. Defibrination of whole blood prior to density gradient centrifugation, high speed centrifugation of plasma and washing procedure reduced platelet contamination to less than 3 × 10⁶ cells/l. Red blood cell contamination was less than 20 × 10⁶ cells/l. No attempts were made to destroy contaminant red blood cells as they were unlikely to contribute appreciably to the measured heat effect and more likely to provide a useful aerobic milieu for the lymphocytes during calorimetric measurements. Lymphocyte preparation and cell counts were completed in 4 to 5 h and all preparation procedures were carried out at 25° C except where stated otherwise.

Calorimetry

Microcalorimeters of the thermopile heat conduction type were used. Samples were enclosed in a 1 ml stainless steel ampoule. The determinations were carried out under static conditions in the type of instrument described in previous reports (Monti & Wadsö 1973, Bandmann et al 1975). The reported heat effects were calculated from readings taken exactly 1 h after the cell suspension had been incubated under static conditions in the calorimeter.

RESULTS

The mean heat production was 2.6 ± 0.5 (SD) pW/cell in the group of healthy subjects and 1.80 ± 0.5 pW/cell in the CLL group (Figure 1) at a pH of 7.78 ± 0.07 and 7.74 ± 0.08 respectively. The difference in heat production is significant (P < 0.005).

DISCUSSION

The heat production by lymphocytes in the group of healthy subjects was found to be

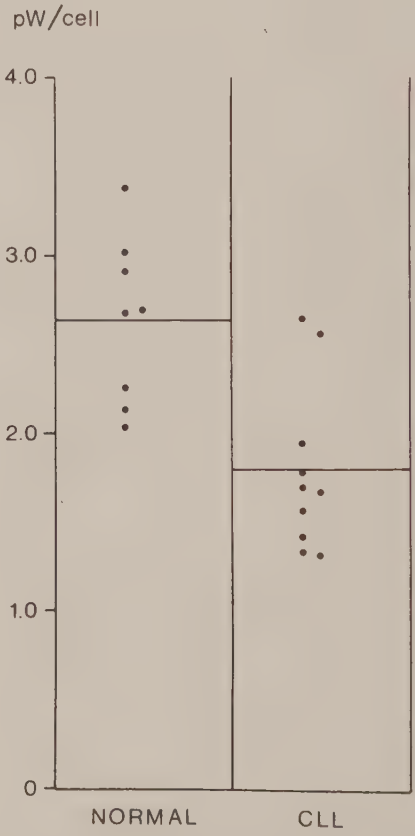


Figure 1. Heat effects of lymphocytes (pW/cell) suspended in plasma. Normal subjects and patients with CLL.

in fairly good agreement with the results previously obtained in a group of normal persons, 2.2 ± 1.4 (SD) (Bandmann et al 1975). In the CLL group the heat production per lymphocyte was found to be sub-normal, indicating a decreased metabolic activity in these cells. This result is in line with the findings of Brody et al (1969) who demonstrated an impaired pentose phosphate shunt and a decreased glycolytic activity in CLL lymphocytes. The reported hypometabolism of the lymphocytes probably does not reflect a generalised reduction of cellular metabolic activity in CLL. Thus a normal and even elevated heat production has been found in red cells (Monti, to be published). Using the mean values for the heat production per lymphocyte obtained in the present study, calculations of the heat production of the intravascular lymphocyte pool in a healthy subject with a peripheral blood lymphocyte count of $2.0 \times 10^9/l$ amount to some 0.03 W. In CLL with a lymphocyte count as high as $200 \times 10^9/l$ the corresponding value is 1.80 W i.e. approximately 60 times higher.

There is evidence that in CLL the intravascular pool of lymphocytes is part of a lymphocyte recirculating pool (LRP) (Schiffer 1968, Stryckmans et al 1977). According to data given by Stryckmans et al (1977) it can be estimated that the LRP in a CLL patient with a blood lymphocyte count of $200 \times 10^9/l$ is about 3.5×10^{10} lymphocytes per kg body weight. From our data on the heat production by the leukaemic lymphocytes it can be calculated that in such patient weighing 70 kg the LRP will produce heat corresponding to about 4 W. There is evidence (Schiffer 1968) that the LRP constitutes only a minute fraction of the total lymphoid mass in CLL and that another compartment, the tissue lympho-

cytes, may constitute the major tumour mass. The heat production of the tissue lymphocytes in CLL is therefore probably several times that calculated for the LRP. The total heat production of the normal human body is about 100 W (Kleiber 1961). The accumulation of lymphocytes in various tissues in CLL therefore means a significant increase in the metabolically active cells contributing to the hypermetabolic state often seen in CLL. Such concept is supported by the finding of a reduction of the basal metabolic rate following splenectomy in CLL patients with considerable splenomegaly (Videbaek et al 1976).

In order to investigate specific cell properties calorimetry has to be combined with other analysis. However, calorimetry has been found to be a useful method to determine the total metabolic activity of various cell populations including red cells, granulocytes, platelets and lymphocytes (Spink & Wadsö 1976, Monti & Wadsö 1978). In the present work calorimetry has been applied to the study of malignant cells. Although such cells are most easily obtained from the peripheral blood in malignant haematologic conditions it is conceivable that calorimetry can be applied to tumour cells obtained through fine-needle aspiration biopsy or to excised material.

ACKNOWLEDGEMENTS

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Metabolic Activity of Lymphoma Cells and Clinical Course in Non-Hodgkin Lymphoma (NHL)

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By microcalorimetry the metabolic activity of malignant cells from 21 patients with non-Hodgkin lymphoma was monitored. Heat production, a measure of the metabolic activity of cells, showed positive correlation with the clinical course of the disease. The heat produced by tumor cells from patients with progressive disease was 5.5 pW/cell, while that from patients responding to treatment was 3.1 pW/cell. The difference between the 2 groups is significant ($P < 0.05$). A higher heat production was obtained in tumor cells from 7 out of 10 patients who were unresponsive to the therapy when compared with cells from 11 patients who had become asymptomatic as a result of the same therapy. Also heat production from blood lymphocytes was found to be higher ($P < 0.005$) in the group of patients with progressive disease as compared to the group of patients who responded to therapy. The present results indicate an association between high heat production in lymphoma cells and blood lymphocytes and a poor prognosis.

Key words: calorimetry – heat production – metabolism – NHL

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Non-Hodgkin (NHL) is a heterogenous group of disorders with varying response to treatment and variable prognosis. Classification based on cell morphology is considered important in the proper management of NHL because morphology correlates to the prognosis. There is, however, controversy about the classification of lymphomas as the clinical course only roughly correlates with the various histologic and cytologic criteria. In the present work, the sensitive method of microcalorimetry was

used to study the relationship between the clinical course and the metabolism of lymphoid cells from patients with NHL.

MATERIAL AND METHODS

Patients. Samples were obtained from 21 patients with NHL treated at the Department of Oncology, University Hospital, Lund. All patients included in the study had palpable lymph nodes accessible to puncture. Apart from this no other selection criteria were adopted. Untreated patients as well as patients pre-

TABLE 1
Clinical data and histology of 21 patients with NHL

Pat. no.	Sex	Age	Diagnosis	Clinical stage	Previous therapy	Puncture site	Later therapy	Clinical course
1	F	63	DH	III	MEV	Axilla	CHOP	Died after 1 mo.
2	M	66	DLPD	II	Radiation	Axilla	CVP	Died after 12 mo.
3	M	75	NH	II	-	Groin	Prednimustine	Partial remission after 15 mo.
4	M	73	DH	IV	-	Arm	MEV	Partial remission after 12 mo.
5	M	66	DH	III	-	Groin	MEV, CHOP	Complete remission after 18 mo.
6	M	47	NM	II	-	Groin	Radiation	Complete remission after 18 mo.
7	M	47	DH	I	Radiation	Groin	Radiation	Complete remission after 12 mo.
8	F	81	DLWD	II	-	Neck	Radiation	Complete remission after 22 mo.
9	M	73	DLPD	III	Radiation	Groin	Radiation	Complete remission after 12 mo.
10	M	65	DM	II	-	Neck	Radiation	Complete remission. Died after 5 mo. Pneumonia.
11	M	67	DLPD	III	PBM, CVP, CHP	Neck	Prednimustine	Died after 4 mo.
12	M	71	DH	IV	Prednimustine	Axilla	Prednisolone, Oncovine	Died after 1 mo.
13	F	46	DH	IV	-	Neck	MEV	Died after 4 mo.
14	M	80	DH	II	-	Neck	MEV, Prednimustine	Died after 17 mo.
15	M	49	DLPD	I	Prednimustine	Groin	Prednimustine	Died after 4 mo.
16	F	80	DLPD	IV	Prednimustine	Neck	Prednimustine, CVP	Died after 5 mo.
17	M	57	DM	II	Radiation, MEV	Groin	Radiation, MEV	Died after 7 mo.
18	F	46	DM	IV	-	Neck, axilla	MEV	Partial remission after 8 mo.
19	F	73	DH	IV	MEV, CHOP, Prednimustine	Groin	Prednimustine	Died after 2 mo.
20	F	62	DLPD	IV	-	Axilla	Prednimustine, CVP	Partial remission after 6 mo.
21	F	42	NM	II	-	Groin	Radiation	Complete remission after 8 mo.

DH = Diffuse histiocytic. NH = Nodular histiocytic. NM = Nodular mixed. DLWD = Diffuse lymphocytic well differentiated. DLPD = Diffuse lymphocytic poorly differentiated. DM = Diffuse mixed. MEV = Methotrexate, cyclophosphamide, vincristine. PBM = Prednimustine, bleomycin, methotrexate. CVP = Cyclophosphamide, vincristine, prednisolone. CHP = Cyclophosphamide, doxorubicin, prednisolone. CHOP = Cyclophosphamide, doxorubicin, vincristine, prednisolone.

viously on chemotherapy were included. All patients were off treatment at the time of the study. The histological diagnosis and clinical data are given in Table 1.

Preparation of samples. Lymphocytes were isolated from defibrinated whole blood by density gradient centrifugation in Ficoll-Paque medium (Pharmacia, Uppsala, Sweden) by the method of Böyum (1968). Lymphoid cells from tumor masses were obtained by repeated (3–6 times) fine needle punctures and the aspirated material was suspended in autologous plasma. The sites from which the tumor biopsies were taken are indicated for each patient in Table 1. Phagocytizing cells were removed by incubating the cell

suspensions in a phosphate buffer containing iron filings and 10% autologous plasma at 37°C under rotation for ½ h and removing the iron adherent phagocytic cells with a magnet. The cells were washed twice in 50 ml phosphate buffer, pH 7.40, and re-suspended in autologous cell-free plasma which had been recentrifuged at 8000 × g. Calorimetric measurements were carried out using cells in the range 0.4–5.7 × 10⁶ and 0.4–5.0 × 10⁶ for blood and lymph node cells, respectively, suspended in 1 ml plasma in a sealed steel ampoule. Cell count in each cell suspension was made in duplicate in a Bürker chamber just before the calorimetric measurements. In a few cases platelets and red blood cells were counted. The same result was obtained as in previous experiments, where the same

preparation technique was used (Brandt et al 1979): platelet contamination was less than 3×10^6 cells/l, red blood cell contamination was less than 20×10^6 cells/l. All the steps involved in cell preparation were carried out at 25°C and lasted 4–5 h. The pH of the final cell suspension taken directly from the calorimeter ampoule at the end of 1 h incubation at 37°C was determined using a capillary electrode pH meter (Radiometer), type G279/G7. Calorimetric measurements were also made on cell-free plasma samples prepared from the same blood samples as used for the measurement of lymphocytes.

Calorimetry. 3 nearly identical twin calorimeters of the thermopile heat conduction type were used. Cell suspension and plasma samples were enclosed in a 1 ml stainless steel ampoule. Calorimetric determinations were carried out under static conditions at 37°C as described previously (Monti & Wadsö 1973, Bandmann et al 1975). The reported heat effects were calculated from power time curves in steady state read exactly 1 h after the cell suspension or plasma had been incubated under static conditions in the calorimeter. Heat effects are reported in terms of watt, $1 \text{ W} = 0.2390 \text{ cal/s}$.

RESULTS

6 months after the whole program of calorimetric measurements was completed a clinical evaluation of the patients' condition was made. The patients were considered to have responded to therapy (remission) when the total tumor volume had decreased during treatment, at least 50%. The results of calorimetric measurements are shown in Figures 1 and 2 where the heat production in blood lymphocytes from 10 healthy persons is also reported, mean 2.7 ± 0.6 (SD) pW/cell ($\text{pW} = 10^{-12} \text{ W}$). Of the 21 patients studied 10 patients had progressive disease despite treatment, 7 of whom expired. In the cell suspension obtained from their lymphomas (Figure 1) the mean heat production per cell was 5.5 ± 3.3 (SD) pW. 1 patient in complete remission (case no 10) died after 5 months from pneumonia unrelated to NHL. The lymphoma cells from

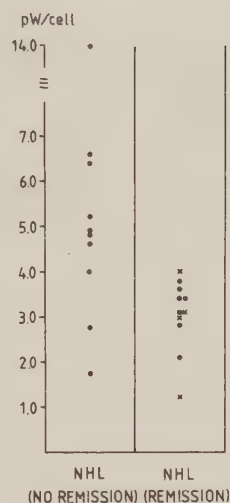


Figure 1. Heat production of tumor cells from NHL patients.

● Patients with no remission and with complete remission.
 × Patients with partial remission.

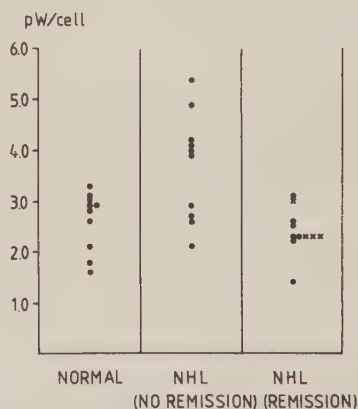


Figure 2. Heat production of blood lymphocytes from NHL patients and normal subjects.

● Normal subjects, patients with no remission and with complete remission.
 × Patients with partial remission.

the remaining patients who were in complete or partial remission had a mean heat production of 3.1 ± 0.8 (SD) pW/cell. The difference between the 2 groups is significant ($P < 0.05$). The mean value for heat

TABLE 2

Heat production in tumor cells, blood lymphocytes and plasma from NHL patients
P_t is the total heat production recorded for cells in plasma suspension and for plasma
P is the heat production calculated after subtraction of heat production in plasma

Pat no	P _t , μW/ml		P _e , μW/ml	P, pW/cell	
	Tumor cells	Blood lymphocytes	Plasma	Tumor cells	Blood lymphocytes
1	3.9	3.5	1.3	4.6	4.0
2	8.4	10.9	1.4	1.7	2.7
3	7.1	4.6	1.9	4.0	2.3
4	2.9	5.3	0.9	1.2	2.3
5	6.4	4.3	2.3	3.1	1.4
6	9.7	13.4	2.6	2.1	3.1
7	7.1	7.9	1.4	3.4	2.2
8	15.1	10.0	1.4	3.4	2.3
9	3.4	10.6	1.6	3.7	2.6
10	14.1	9.8	0	2.8	2.3
11	8.9	7.5	1.3	6.4	5.4
12	4.1	13.4	1.4	2.8	2.1
13	6.0	14.0	0	14.0	3.9
14	3.7	12.7	1.4	6.6	2.9
15	8.2	4.3	1.0	4.8	2.6
16	6.8	6.2	-1.9	4.0	4.1
17	2.6	3.1	0.9	5.2	4.9
18	5.1	7.8	3.2	3.1	2.3
19	4.4	11.1	2.0	4.9	4.2
20	5.2	5.2	3.4	3.0	3.0
21	4.9	11.3	2.9	3.6	2.5

TABLE 3

Mean heat production values (pW/cell ± SD) of tumor cells and blood lymphocytes from NHL patients according to their histological classification

	TUMOR CELLS	BLOOD LYMPHOCYTES
HISTIOCYTIC	5.0 ± 3.7	2.6 ± 1.2
MIXED	3.4 ± 1.2	3.4 ± 2.0
LYMPHOCYTIC	3.9 ± 1.5	3.2 ± 1.1

production per lymphocyte from peripheral blood (Figure 2) was 3.7 ± 1.1 (SD) pW in the group of patients who did not respond to therapy. In the group of patients with remission the corresponding pretreatment value was 2.4 ± 0.5 (SD) pW/cell. The difference between the 2 groups is statistically significant (P < 0.005). All values reported have been corrected for the amounts of heat produced by plasma (Table 2). Cell viability was not tested. How-

ever, in another group of NHL patients under current investigation and not belonging to the present study, but using the same method of cell preparation as in this study, viability was determined by trypan blue exclusion test and found to be 90% ± 8% (SD) for lymphoid cells from tumor masses, and 97% ± 2% for blood lymphocytes. No correlation was found between cell heat production and viability index (percentage of viable cells). The number of erythrocytes and platelets in the cell suspension was too low to give a significant contribution to the heat production reported. pH values in the cell suspension at the end of the calorimetric experiments were close to those reported previously in similar experiments, i.e. about 7.80 (Brandt et al 1979), and there was no significant pH difference between the 2 groups of cell suspension. In

the healthy control group pH in the cell suspension was also about 7.80. The mean heat production values were also calculated for the different groups of patients according to their histological classification (Table 3): histiocytic, mixed, lymphocytic.

DISCUSSION

In the present study the metabolic activity in lymphoma cells and blood lymphocytes of 21 NHL patients was measured by a microcalorimetric technique, recently introduced for monitoring metabolic processes in various cell systems, particularly blood cells (Monti & Wadsö 1979). The heat production in lymphoid cells aspirated from tumor masses was higher in 7 out of 10 patients who did not respond to treatment than in 11 other patients with a response to therapy. The period of observation was short and the number of patient is too small to allow conclusions about the survival rate. However, 7 out of 10 patients in the first group expired during the observation period. The heat production in tumor cells and blood lymphocytes was not found to be significantly different ($P \geq 0.05$) between the 3 groups of patients classified according to their histological diagnosis.

Most NHL derive from B-cells (Leech et al 1975, Payne et al 1977). It has been demonstrated that the glycolytic metabolism is considerably higher in B-lymphocytes than in T-lymphocytes (Storch et al 1978). A predominance of B-cells in the tumors might therefore be a contributory factor for the high heat production in the cells obtained from enlarged lymph nodes. However, it is worth noting that the heat production of tumor cells from patients who responded to treatment was of the same magnitude as that obtained from normal

peripheral blood lymphocytes. Moreover, in chronic lymphocytic leukemia, which is generally a B-cell lymphoproliferative disease, the heat production in peripheral blood lymphocytes was lower than that in lymphocyte suspensions from healthy subjects (Brandt et al 1979). We believe therefore that the difference in heat production in cell suspensions obtained from lymphomas is not due to variations in the B-cell content. It is more probable that the differences recorded depend on variations in metabolic activity between different B-cell tumors.

Increased heat production in blood lymphocytes was noted in 6 out of 10 patients in the group not responding to treatment. Using immunologic surface markers it has been demonstrated that in NHL malignant lymphoid cells are often present in the peripheral blood (Garrett et al 1979). It is possible that the increased heat production in peripheral blood lymphocytes of patients who did not respond to treatment in this study was due to a spilling of metabolically highly active lymphoma cells into the circulation.

Our results suggest that a high metabolic activity in lymphoma cells is associated with an aggressive growth of the tumor tissue. It has been reported that high levels of serum lactic dehydrogenase (LHD) is related to a poor prognosis in NHL (Ferraris et al 1979). These findings may support the view that lymphomas consisting of metabolically very active cells may be especially aggressive.

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